Isolation, identification, screening of toxicity and oligopeptides of some marine and brackish cyanobacteria from Norwegian and Pakistani waters, in the search for bioactive natural compounds

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DEDICATION

Dedicated to my beloved sister, Fahmida Hameed
and her children.

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I wish to express my thanks for the guidance of. Prof. Dr. Bente Edvardsen, University of Oslo (UiO), Norway. I acknowledge my supervisor for her constant encouragement and kind supervision throughout the period of research. I am also thankful to my co-supervisor Dr. Thomas Rohrlack (Research Scientist) at Norwegian Institute of Water Research (NIVA) to work on LC-MS analyses of cyanobacterial strains. My special and cordial thanks for Linn Oftedal (University of Bergen, Norway)

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ABSTRACT

Cyanobacteria produce a number of bioactive compounds, most of them are oligopeptides. Almost all are known from freshwater species. The aim of this study was to search for marine and brackish water species producing bioactive compounds. To reach this goal, new strains were isolated from Norwegian and Pakistani coastal waters. These and additional strains from NIVA, UiO and UiB culture collections (24 in total), belonging to Chroococcales and Oscillatoriales, were identified based on morphology and sequencing of the partial *cpc*BA phycocyanin DNA and partial 16S rDNA-regions. Their systematic position was determined by phylogenetic analyses. The bioactivity was tested by *Artemia* (brine shrimp) bioassay, and the presence of potentially bioactive oligopeptides was determined by LC-MS/MS. All sequences in this study, except of one strain, clustered with known sequences of *Geitlerinema* spp., *Phormidium* spp., *Pseudoanabaena* spp. and *Synechococcus* spp. with high support. *Oscillatoria* sp. UIO 017, did however not cluster with any sequence in gene databases. Genetic analyses showed that strains from Norway of the genus *Synechococcus* divided into 3 clades, suggesting cryptic species. Only one strain, *Geitlerinema* sp. UK-G-106 was lethal to *Artemia* nauplii. The combined concentrations of crude extract of *Geitlerinema* sp. UK-G-106 showed LC_{50}-24 h 0.0032 mg dw mL^{-1} and was more toxic than toxic freshwater strains *Planktothrix rubescens* (LC_{50}-24 h 0.05 mg dw mL^{-1}), *P. agardhii* (LC_{50}-24 h 0.06 mg dw mL^{-1}) and *Microcystis aeruginosa* (LC_{50}-24 h 0.12 mg dw mL^{-1}). The fractionation of solid phase extractions (methanol:water) of crude extract of *Geitlerinema* sp. UK-G-106 revealed that the percentage of mortality decreased as concentration of methanol increased. The highest LC_{50}-24 h 0.15 mg dw mL^{-1} was found at 0% MeOH.

LC-MS/MS analysis of *Geitlerinema* sp. UK-G-106 showed the presence of unknown oligopeptides. These compounds may be screened for additional bioactivity in the search for new therapeutical drugs.

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1. INTRODUCTION

1.1. Cyanobacterial occurrence

Cyanobacteria (Cyanoprokaryota, Cyanophyta, blue green algae) are belong to an ancient and diverse group of organisms that evolved in the middle of the Pre-Cambrian era, approximately 2,600 to 3,500 million years ago (Myr) based on fossil records (Schopf 2000), organic biomarkers (Brocks *et al* 1999) and genomic sequence analysis (Hedges *et al* 2001). The earliest estimate of cyanobacterial occurrence is 3,500 Myr (Brasier *et al* 2002). They are Gram-negative (Gerba *et al* 2000) photoautotrophic prokaryotes (without nucleus) having higher plant-type oxygenic photosynthesis (Whitton and potts 2000). They are believed to be responsible for the oxygenation of the earth's atmosphere around one billion years after their appearance in the fossil record (Catling *et al* 2001, Kasting 2001). Cyanobacteria in the global ecosystem are very important and it is believed that the marine cyanobacteria *Synechococcus* and *Prochlorococcus* carry out 32-88 % of primary production in the oligotrophic ocean (Rocap *et al* 2002).

Cyanobacteria are commonly found in an extremely broad range of environments including hot springs, freezing Antarctic lakes (Singh and Elster 2007 and Comte *et al* 2007), glaciers (Mur *et al* 1999), soils (Bhatnagar *et al* 2008), extreme hypersaline environments, sea bottoms (Lopez-Cortes *et al* 2005), alkaline lakes (Ballot *et al* 2005), eurythermal and habitats of fresh and marine waters. They also form biofilms (microbial mats) on shores, mangrove swamps and on the surface of stones, plants, and artificial objects (Stal 2000). Cyanobacteria have the ability to survive in a wide range of temperatures, (-10-72 ºC; Graham and Wilcox 2000, Lopez-Cortes *et al* 2005, Singh and Elster 2007).

Cyanobacteria may have different colors like olive-green, grey-green, yellow-brown, purple or red and bright green (Ressom *et al* 1994). These organisms are found in a variety of shapes and arrangements from unicellular cocci, oval or rods or long trichomes (Whitton and Potts 2000). In many cyanobacterial species gas vacuoles are found (Paerl 2000). The presence of these vacuoles helps regulate their position in the water column and given them distinct ecological advantage over other planktonic species. Usually unicellular cyanobacteria are non-motile, while filamentous forms generally possess gliding or oscillating motility. The blue-green algae mainly contain chlorophyll *a* and phycobiliproteins (phycocyanin,

allophycocyanin and phycoerythrin), xanthophylls, β-carotene in their photosynthetic cellular constituents and carry out photosynthesis with the production of oxygen (Whitton and Potts 2000, Castenholz 2001a). Some cyanobacteria have evolved specialized cells for nitrogen fixation (heterocytes), survival in stressed conditions (akinetes), and dispersion (hormogonia). In addition, cyanobacteria form symbiosis with several eukaryotic hosts such as plants, fungi, and protists (Adams 2000). In cyanobacteria reproduction can be done by vegetative binary fission, hormogonia, akinetes and fragmentation and asexual spores. Sexual reproduction by gametes is completely absent, but DNA exchange occurs by parasexual processes i.e. transformation, transduction and conjugation.

A dilemma exists as to whether cyanobacteria should be classified under the International Code of Botanical Nomenclature or the International Code of Nomenclature of Bacteria (Skulberg *et al* 1993). These differences led to the taxonomic treatment of these organisms as a separate and distinct group of algae: Class Cyanophyceae. Oren (2004) also proposed further integration of the cyanobacteria under the Bacteriological Code.

1.1.1. Cyanobacterial research in Pakistan

Pakistan has a ca. 1,046 kilometer (ca. 650 miles) coastline along the Arabian Sea in the south and only two provinces (Sindh and Balochistan) are bordered by the Northern Arabian Sea. In the coastal waters of Pakistan a total of 121 species pertaining to 37 genera of cyanobacteria have been reported (RETA and IUCN report 2000). Only single report on phycochemistry and bioactivity of *Microcystis aeruginosa* from Miani Hor, Pakistan was reported by Aftab and Shameel (2006). Another study was made by Bano and Siddiqui (2004) on salinity and pH requirements of some marine cyanobacteria of the rocky shore of Buleji, Pakistan.

In Pakistan some information is available on cyanobacteria from a Lake Kerli and Manora Channel, an extension of Karachi harbor (Codd *et al* 2005). In Pakistan no work has been reported on isolation and purification of cyanobacterial compounds. Some work has been restricted to taxonomy (Bano 1998).

1.1.2. Cyanobacterial research in Norway

Norway is bordered by the Norwegian Sea, the North Sea, Skagerrak and the Barents Sea. It has a coast line from south to north (ca. 1800 km) with fjords (>20,000 km). They have variable natural habitats, which provide suitable geological condition for growth of various microalgae (Edvardsen *et al* 2004). In Norway extensive studies have been done on the monitoring of marine micro-algal blooms, taxonomy, isolation of compounds and bioactivities (Skulberg 2005). Much work has also been done on effects of abiotic factors on production of bioactive oligopeptides produced by the freshwater cyanobacterium *Planktothrix agardhii* Rohrlack and Utkilen (2007) and *Planktothrix* spp. and their bioactive peptides (Halstvedt *et al* 2008). Some work has also been done on seasonal dynamics and depth distribution of *Planktothrix* spp. in Lake Steinsfjorden, Norway (Halstvedt *et al* 2007, 2008). Rudi *et al* (2000) reported DNA sequence information for the small-subunit rRNA gene (16S rDNA) obtained from cyanobacterial cultures collected from different lakes of Norway.

1.2. Ecological importance of cyanobacteria

The marine cyanobacteria *Prochlorococcus* and *Sycnechococcus,* are the most abundant photosynthetic organisms on earth (Rocap *et al* 2002, Bouman *et al* 2006, Penno *et al* 2006). Cyanobacteria are also involved in the global carbon and nitrogen cycles. The biomass of phytoplanktons in surface waters ranges from 40-50 Pg C y^{-1} (1 Pg is equivalent to 10^{15} g). Cyanobacteria fix atmospheric carbon dioxide (CO_2) through photosynthesis and form organic compounds. Hence the high abundance of planktonic cyanobacteria in oceans influence on the global carbon budget. The oceanic primary production gives a total primary production on earth *c.* 10^{16} g C y^{-1} (Kaiser *et al* 2005). In addition, cyanobacteria can also fix dinitrogen, thus play a significant role in providing biologically available nitrogen to the environment (Castenholz 2001b). Cyanobacteria add organic matter to the soil and fix atmospheric nitrogen. They provide nitrogen (in form of NH_4 and/or amino acids, produced through N_2 fixing) that can be utilized by the higher plants (Paerl 2000) like the marine cyanobacterium *Trichodesmiuim* spp. that play an important role in fixation of nitrogen in marine waters (Mulholland and Capone 2000). Cyanobacteria are also important in biodegradiation of oil in oil spill areas (Raghukumar *et al* 2001, Chaillan *et al* 2006).

There is a great potential of cyanobacterial and microalgal biomass for production of useful biochemicals (Fatima and Venkataraman 1999). Cyanobacteria are also known to produce a variety of bioactive compounds of which some have toxic effects and are called cyanotoxins (Duy *et al* 2000, Burja *et al* 2001, Skulberg 2005, Ballot *et al* 2005, Dietrich 2005, Matsunaga *et al* 2005, van Apeldoorn *et al* 2007). Some of these compounds are toxic for many zooplanktons, fish (Bury 2007), turtles (Nasri *et al* 2008), domestic animals, birds, rats (Codd *et al* 1998, Carmichael 2001), wildlife (Dow and Sowboda 2000) and even human beings (Dow and Sowboda 2000, Fleming *et al* 2002, Bischoff and Ramaiah 2007, Osborne *et al* 2008) in fresh (Ibelings and Chorus 2007), brackish (Review by Dittmann and Wiegand 2006) and marine waters (Al-Sabi *et al* 2006, Rodriguez-Salvador 2007). Some metabolites are cytotoxic, and show promise as killers of bacteria, fungi, insects, plants and algae (Tubaro and Hungerford 2007). Some may even serve as agents for attacking tumor cells and viruses (Review by Dittmann and Wiegand 2006). Several reports have suggested that these natural compounds may be used directly in drug industries (Tan 2007), where they may be synthesized for medical use (Proksch *et al* 2002, Gerwick 2003, Dietrich and Hoeger, 2005, Tan 2007). A number of extracts of cyanobacteria are found to be remarkably active against the HIV virus (Schaeffer and Krylov 2000). Cyanobacteria are widely used as food and feed supplement (e.g. *Spirulina* sp.) throughout the world (Gorham and Carmichael 1979).

1.3. Cyanobacterial blooms and their effects

Several species of cyanobacteria can grow abundantly under favourable natural environmental conditions and form high biomass called water blooms which often is associated with eutrophication (Kanoshina *et al* 2003, Milan 2007). Cyanobacterial blooms commonly occur in many temperate lakes (ISOC-HAB 2008, Hudnell *et al* 2008) and also in coastal areas (Vargas-Montero and Freer 2004, Albert *et al* 2005, Watkinson *et al* 2005, Penno *et al* 2006, Hernández-Becerril *et al* 2007, Garczarek *et al* 2007, Dietrich *et al* 2008). The blooms may initially appear green and later turn blue-green, sometimes forming a ‘scum’ on the water surface. These blooms are considered a natural phenomenon, but in recent years their frequency has increased considerably (Kanoshina *et al* 2003, Carmichael 2008). Agricultural runoff and other effluents to fresh and marine water bodies and wetlands have resulted in increased nutrient enrichment of phosphorous and nitrogen (Kanoshina *et al*

2003), thus providing favorable conditions for the growth of toxic cyanobacteria (Sivonen and Jones 1999, Codd *et al* 2006). A range of bloom-forming freshwater and marine cyanobacterial strains are capable to produce toxins and other bioactive compounds (Carmichael, 2001, Welker and von Döhren 2006, Westberry and Siegel 2006, Dietrich *et al* 2008).

Most of the harmful effects of cyanobacterial blooms have been reported from freshwater ecosystems. Several cyanobacteria blooms have also been reported from brackish and marine waters and may have harmful effects on humans (Mira 2005, Hernández-Becerril 2007) and animals (Luckas *et al* 2005, Arthur *et al* 2006, Stewart *et al* 2008). The bloom of a marine cyanobacterium, *Trichodesmium erythraeum* cause sickness, dermatitis and other discomforts (Vargas-Montero and Freer 2004) when bathing in contaminated water. The effects of some harmful algal blooms are not related to toxin production, but they are involved in depletion of dissolved oxygen concentration caused by algal proliferation, death and decay, or night respiration.

1.4. Types and nature of cyanotoxins

Francis (1878) presented the first description on toxicity due to blue-green algae. He described the death of sheep, cattle, dogs, horses and pigs around the estuary of the Murray River, Australia. The main cyanobacterium, which was responsible for these deaths, was *Nodularia spumigena*. Ever since, scientists have been interested in studying the potential lethal effects of cyanotoxins. There are several worldwide fatal poisoning of domestic and wild animals, birds, finfish, shellfish that have been reported (GEOHAB report 2001). According to the mode of action cyanobacterial toxins can be divided into three major categories.

1.4.1. Hepatotoxins

Several freshwater, brackish water and marine cyanobacterial species of the genera *Microcystis, Anabaena, Nodularinria, Oscillatoria, Nostoc* and *Hapalosiphon* (Terrestrial genera; Rinehart *et al* 1994) produce hepatotoxins (Welker 2008, Tango and Butler 2008). These toxins include cylindrospermopsins and peptides.

A. Cylindrospermopsins (CYN) are guanidine tricyclic alkaloid hepatotoxins (CYN; van Apeldoorn *et al* 2007; Fig. 1) and have been isolated from several cyanobacterial species and strains (Table 2). They have a molecular weight of 425 daltons and cause hepatoenteritis.

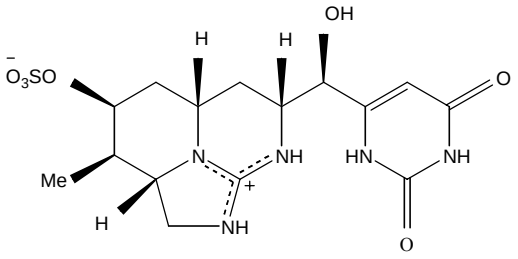


Fig.1. Chemical structure of cylindrospermopsin (Ohtani *et al* 1992)

CYN is genotoxic and cytotoxic and may also cause liver, kidney and other organ damage (Chorus 2005).

B. Cyanobacterial cyclic peptides

On the bases of molecular structures and source of origion cyanobacterial peptides can be divided into 8 main classes (Welker and von Döhren 2006; Table 1).

a. Aeruginosins are linear peptides and characterized by a derivative of hydroxy-phenyl lactic acid (Hpla) and an arginine derivative (Welker and von D□hren 2006; Fig. 2A). Table 1 shows the details of aeruginosins and its producing cyanobacteria.

b. Microginin is a class of linear peptides first described by Okino *et al* (1993a) characterized by a decanoic acid derivative, 3-amino-2-hydroxy-decanoic acid (Ahda) and a predominance of two tyrosine units at the C-terminus (Table 1; Fig. 2B).

c. Anabaenopeptins are cyclic peptides and have been reported from cyanobacteria (Table 1; Fig. 2C), isolated from freshwater (Harada *et al* 1995), terrestrial (Reshef and Carmeli 2002) and brackish waters (Fujii *et al* 1997).

d. Cyanopeptolins these cyclic peptides have high structural variability. Cyanopeptolin type peptides have been isolated from Chroococcales, Oscillatoriales and Nostocales (Table 1; Fig. 2D).

e. Nodularin (NODLN) is a hepatotoxin and its structure was first established by Sivonen *et al* (1989b) as mono cyclic pentapeptide (m/z 824 daltons; Fig. 2E) and reported by Francis (1878). It has been reported from the filamentous brackish water cyanobacterium, *Nodularia spumigena* (Table 2).The chemical structure of NODLN is cyclo-(D-MeAsp^{1}-L-arginine^{2}-Adda^{3}-D-glutamate^{4}-Mdhb^{5}), in which Mdhb is 2-(methyleamine)-2-dehydrobutyric acid. This toxin has also been isolated from the marine sponge *Theonella swinhoei*, which is known to form symbiotic relationships with cyanobacteria (DeSilva *et al* 1992).

f. Microcystins (MC) are the largest cyanobacterial hepatotoxic peptides (molecular weight 1000 daltons; Kuper-Goodman *et al* 1994a, b; Fig. 2F) and were first isolated from the cyanobacterium, *Microcystis aeruginosa* (Bishop *et al* 1959; Table 2). MCs are monocyclic heptapeptides with a common structure to NODLN, containing three D-amino acids (alanine, erythro-β-methylaspartic acid and glutamic acid), two variable L-amino acids, and two unusual amino acids (N-methyldehydroalanine, and 3-amino-9- methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 4-dienoic acid, Adda; Botes *et al* 1985). The authors also reported the structure of four other related toxins having L- amino acid combinations of -LR (lucine-arginine), -YR (tyrosine-arginine), -YA (tyrosine-alanine) and -YM (tyrosine-methionine). More than 67 MCs and their variants have been characterized from blooms and isolated strains of cyanobacteria. The cyclic peptide MC, NODLN and guanidine alkaloid CYN are specific liver poisons in mammals. Following acute exposure to high doses, they cause death from liver haemorrhage or failure, and they may promote the growth of the size of liver and may also result in tumors forming following chronic exposure to low doses (Clark *et al* 2007). MC and NODLN may also cause haemodynamic shock, heart failure consequently death. In other animals they have affected the kidneys, lungs (Henriksen 2005) and intestines (Falconer and Humpage 1996).

Table. 1. General features of cyanobacterial peptides (selected references).

Oliopeptides	Type of peptide	Microalgal origin	References
I. Main classes of peptides			
Aeruginosins	Linear peptide	*Microcystis, Planktothrix, Nodularia*	Murakami *et al* 1995
a. Suomilide		*Nodularia*	Fujii *et al* 1997
b. Banyaside		*Nostoc*	Ploutno and Carmeli 2005
Microginins	Linear peptide	*Microcystis, Planktothrix, Nostoc*	Okino *et al* 1993a, Ishida *et al* 2000, Ishida *et al* 1998
a. Nostoginin		*Nostoc*	Ploutno and Carmeli 2002
Anabaenopeptins	Cyclic peptide	*Anabaena, Aphanizomenon. Microcystis, Planktothrix*	Harada *et al* 1995, Reshef and Carmeli 2002, Fujii *et al* 1997
Cyanopeptolins	Cyclic peptide	*Anabaena, Lyngbya, Microcystis, Planktothrix, Scytonema, Symploca, Cdroococcales, Oscillatoriale Nostocales*	Harrigan *et al* 1999, Martin *et al* 1993, Okino *et al* 1993b, Tsukamoto *et al* 1993, Harada *et al* 1993
Microcystins	Cyclic peptide	*Microcystis, Anabaena, Planktothrix, Nodularia Nostoc, Hapalosiphon, Anabaenopsis*	Botes *et al* 1984 (see more references in Table 2)
Nodularins	Cyclic peptide	*Nodularia*	Sivonen *et al* 1989b (see more references in Table 2)
Microviridins	Multicyclic peptide	*Microcystis, Planktothrix, Nostoc*	Ishitsuka *et al* 1990,
Cyclamides	Cyclic hexapeptide	*Lyngbya, Microcystis, Nostoc, Oscillatoria, Stigonema, Westelliopsis*	
a. Nostocyclamide		*Nostoc*	Todorova *et al* 1995
b. Westiellamide			Prinsep *et al* 1992
II. Other peptides			
Crytophycins	Cyclic desipeptide	*Noctoc*	Schwartz *et al* 1990
Microcolins	Linear peptide	*Lyngbya*	Koehn *et al* 1992
Mirabimids	Linear peptide	*Scytonema*	
Tantazoles	Linear tetrapeptide	*Scytonema*	Carmeli *et al* 1990 and 1991
Mirabazoles	Pentapeptide	*Scytonema*	Carmeli *et al* 1990 and 1991
Other peptides			
a. Aeruginosinamide	Linear tetrapeptide		Lawton *et al* 1999
b. Barbamide	Linear tetrapeptide	*Lyngbya*	Orjala and Gerwich 1996, Williamson *et al* 1999
c. Lyngbyabellin B	Cyclic hexapeptide	*Lyngbya*	Luesch *et al* 2000a
d. Apramides	Linear nonapeptide		Luesch *et al* 2000b
e. Wewekazole	Cyclic undecapeptide	*Lyngbya*	Nogle *et al* 2003
f. Puwainaphycin	Cyclic deca- and undecapeptide	*Anabaena*	Gregson *et al* 1992
h. Laxaphycin	Lipopeptide	*Anabaena*	Frankm□lle *et al* 1992a, b
i. Lobocyclamide			MacMillan *et al* 2002
j. Calophycin		*Calothrix*	Moon *et al* 1992
k. Kawaguchipeptin	i. Cyclic deca- and undecapeptide		
	ii. Undecapeptide	*Microcystis*	Ishida *et al* 1996 and 1997
l. Oscillatorin	Cyclic decapeptide		Sano and Kaya 1996
m. Radiosumin			Matsuda *et al* 1996
n. Aeruginoguanidin	Tripeptide		Ishida *et al* 2002
o. Kasumigamide	Linear pentapeptide		Ishida and Murkakami 2000
p. Antanapeptin		*Lyngbya*	Nogle and Gerwich 2002
q. Malevamide C	Mono-cyclic peptide		Horgen *et al* 2000
r. Yanucamide		*Lyngbya/Schizothrix* assemblage	Sitachitta *et al* 2000

(based on Welker and von Dohren 2006)

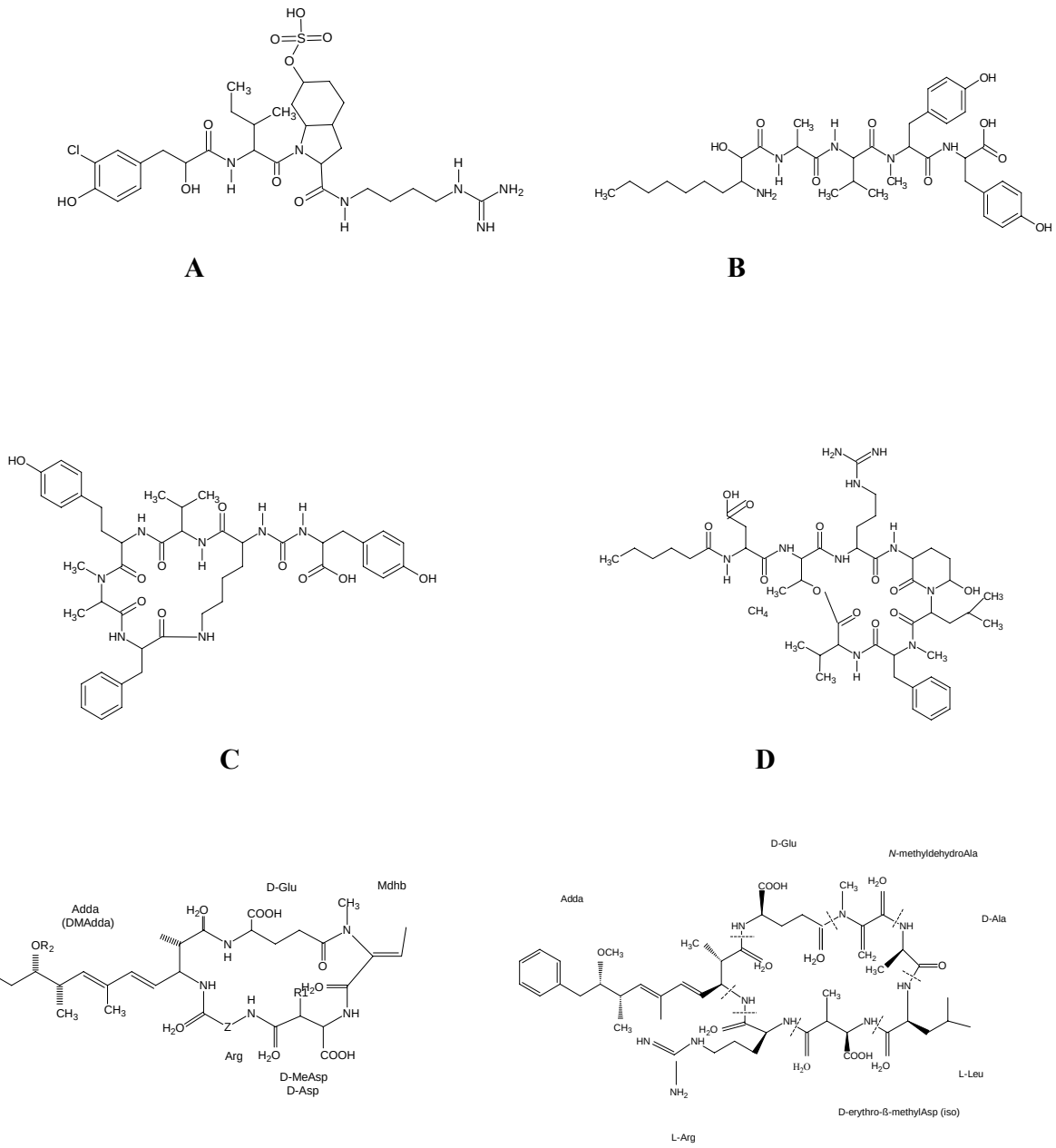
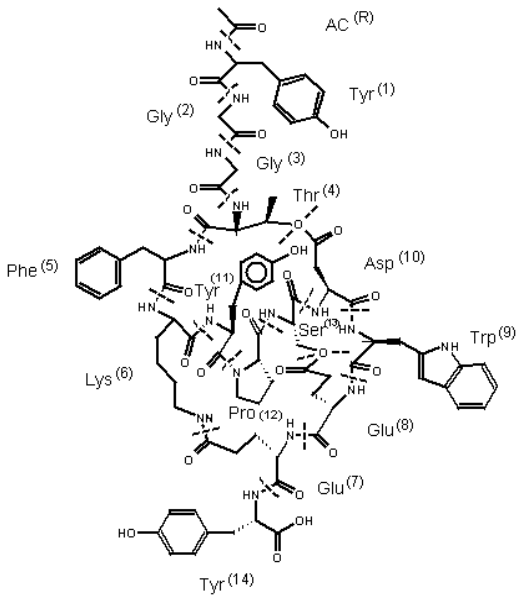
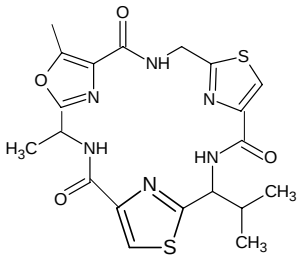


Fig. 2. General structure of cyanobacterial peptides. A) Aeruginosin 98-A (Mukrakami *et al* 1995), B) Microginin (Okino *et al* 1993a), C) Anabaenapeptins A (Harada *et al* 1995),
D) Cyanopeptolin (Martin *et al* 1993), E) Nodularin (Sivonen *et al* 1989b), F) Microcystin (Bishop *et al* 1959).





G **H**

Contd….Fig. 2

G) Microviridin A (Ishitsuka *et al* 1990) and H) Nostocyclamide (Todorova *et al* 1995).

g. Microviridins have also isolated from many cyanobacterial strains (Table 1). The main peptide ring consists of seven amino acids with an ester bond (Fig. 2G and; Ishitsuka *et al* 1990).

h. Cyclamides also have various structures and isolated from several strains of cyanobacteria (Fig. 2H; Table 1).

In addition to these cyanobacterial peptides a variety of more rare peptides have been reported from various species of cyanobacteria listed in Table 1.

1.4.2. Neurotoxins

They are alkaloid in nature and five neurotoxins have been studied in detail (Table 2). About 46 cyanobacterial species are able to produce neurotoxins, e.g. anatoxin-a, anatoxin-a(s) and saxitoxin (Ernst *et al* 2006).

a. Anatoxin-a (AnTx-a) formerly called 'very fast death factor' (VFDF) is a bicyclic secondary amine of alkaloid origin (Fig. 3A). This toxin was first described in the freshwater

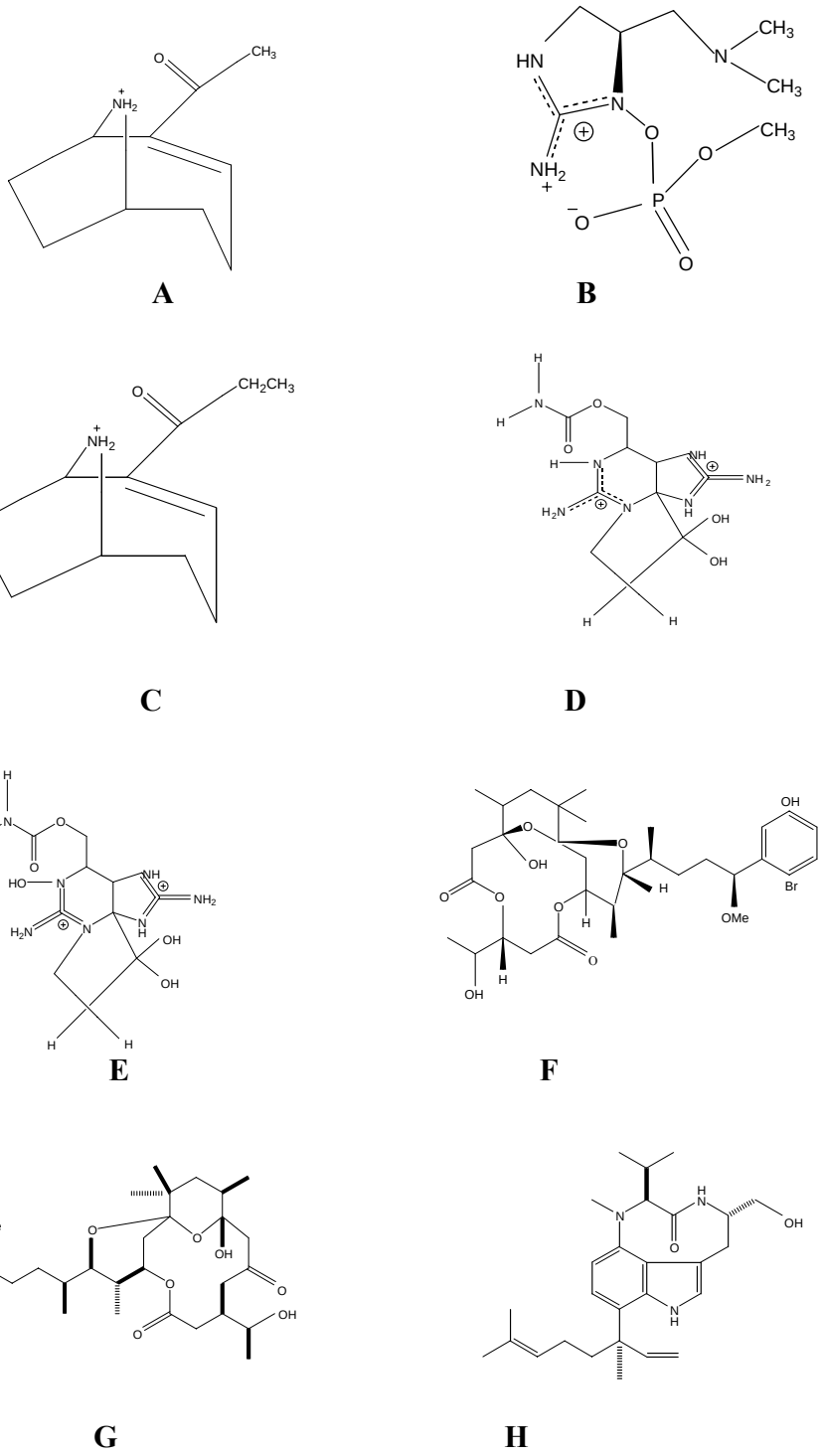


Fig. 3. General chemical structures of cyanobacterial neurotoxins. A) Anatoxin-a (Huber 1972), B) Anatoxin-a (s) (Matsunaga *et al* 1989), C) Homoanatoxin-a (Skulberg *et al* 1992), D) Saxitoxin (Sawyer *et al* 1968) and E) Neosaxitoxin (Sawyer *et al* 1968) and skin irritants: F) Aplysiatoxin (Mynderse *et al* 1977), G) Debromoaplysiatoxin (Cardellina *et al* 1979) and H) Lyngbyatoxin (Mynderse *et al* 1977).

Table. 2. General features of the biotoxins of cyanobacterial species (selected references).

Toxic group	Primary target cells/organs in mammals	Microalgal genera and species	References
Cyclic peptides (hepatotoxin)			
Microcystins (MC)	Liver Gastrointestinal illness	*Microcystis aeruginosa*	Bishop *et al* 1959, Konst *et al* 1965, Lippy and Erb 1976, Elleman *et al* 1978, Botes *et al* 1984 and 1985, Gathercole and Thiel 1987, Watanabe *et al* 1988, Stoner *et al* 1989, Kaya and Watanabe 1990, Sivonen *et al* 1992a,b,c, Craig *et al* 1993, Azevedo *et al* 1994, Bateman *et al* 1995, Lahti 1997
		Microcystis spp.	Yu *et al* 1988, Namikoshi *et al* 1992, Namikoshi *et al* 1995, Luukkainen *et al* 1994, Lahti 1997
		Anabaena sp.	Harada *et al* 1991b, Sivonen *et al* 1992a, Namikoshi *et al* 1992 Namikoshi *et al* 1995, Namikoshi *et al* 1998
		Microcystis wesenbergii	Botes *et al* 1985, Carmichael *et al* 1988, Luukainen *et al* 1993
		Microcystis viridis	Harada *et al* 1990, Barco *et al* 2002
		Planktothrix, Nostoc, Hapalosiphon,	Lawton *et al* 1994, Sivonen and Jones 1999, Ressom *et al* 1994, Briand *et al* 2003
		Anabaen, Planktothrix, Anabaenopsis milleri	Namikoshi *et al* 1992, Namikoshi *et al* 1998, Chorus and Bartram 1999, Sivonen and Jones 1999, Diehnett *et al* 2005, Agrawal *et al* 2006
		Planktothrix agardhii	Krishnamyrthy *et al.* 1989, Luukkainen *et al.* 1993
		Anabaena flos-aquae	Harada *et al* 1991a, Sivonen *et al* 1992d
		Nostoc sp.	Sivonen *et al* 1990a, Namikoshi *et al* 1990, Sivonen *et al* 1992c, Beattie *et al* 1998
		Nodularia spumigena AV1	Fujii *et al* 1997
Nodularin (NODLN)	Liver	*Nodularia spumigena*	Francis 1878, Lindstrøm 1976, Main *et al* 1977, Persson *et al* 1984, Edler *et al* 1985, Eriksson *et al* 1988, Carmichael *et al* 1988, Baker and Humpage 1994, Runnegar *et al* 1988, Rinehart *et al* 1988, Sivonen *et al* 1989b, Jones *et al* 1994

Contd……

Table 2. Contd……

Guanidine alkaloid (hepatotoxin)		*Nodularia* PCC 7804	Beattie *et al* 2000
Cylindrospermopsin (CY)	Liver	*Cylindrospermopsis rasciborscii*	Hawkins *et al* 1985
		Anabaena bergii	Schembri *et al* 2001
		C. ovalisporum	Shaw *et al* 1999
		Planktothirx, Raphidiopsis, Microcystis, Anabaena	Carmichael 1978, Al-Lay *et al* 1988, Carmichael *et al* 1988
		Nodularia, Lyngbya and *Nostoc*	Krishnamurthy *et al* 1989, Harada *et al* 1991a and b, 1994, Ohtani *et al* 1992 Banker *et al* 1997, Hawkins *et al* 1997, Schembri *et al* 2001 and Li *et al* 2001, Fastner *et al* 2003
		Lyngbya wollei	Seifert *et al* 2007
		Aphanizomenon onalisporum	Shaw *et al* 1999
		Aphanizomenon ovalisporum	Banker *et al* 1997, Shaw *et al* 1999
		Aphanizomenon flos-aquae	Preußel *et al* 2006
		Cylindrospermopsis raciborskii	Hawkins *et al* 1985 and 1997, Törökné 1997
		Umezakia natans	Harada *et al* 1994
(Neurotoxin)			
Anatoxin-a (AnTx)	Nerve synapse Depolarize neuromuscular blocking agent	*Anabaena palnktonica, Cylindrospermum* sp.,	Gorham *et al* 1964, Carmichael *et al* 1975 and 1990, Devlin *et al* 1977
		Phormidium favosum,	Carmichael and Bent 1981, Sivonen *et al* 1989a, Edwards *et al* 1992,
		Anabaena planktonica,	Rapala *et al* 1993, Bruno *et al* 1994, James *et al* 1997a, b
		Raphiidiopsis mediterranea,	Namikoshi *et al* 2003, Viaggiu *et al* 2004, Gugger *et al* 2005, Ballot *et al* 2005
		Planktothrix rubescens, Anthrospira fusiformis	Viaggieu *et al* 2004
		Anabaena flos-aquae	Gorham *et al* 1964, Hurber 1972, Carmichael *et al* 1975, Devlin *et al* 1977, Carmichael 1992, Carmichael and Bent 1981, Rapala *et al* 1993,
		Anabaena spp.	Carmichael 1992, Carmichael *et al* 1975
		Anabaena blooms	Sivonen *et al* 1989a, James *et al* 1997a , b
		Anabaena planctonica bloom	Bruno *et al* 1994, Sivonen *et al* 1989a
		Anabaena circinalis	Sivonen *et al* 1989a, Bruno *et al* 1994, James *et al* 1997a, b
		Anthrospira fusiformis	Ballot *et al* 2005
		Aphanizomenon sp.	Codd *et al* 1997, Sivonen *et al* 1989a
		Aphanizomenon blooms	Bumke-Vogt 1999
		Anabaena spiroides	Carmichael 1992
		Cylindrospermum sp.	Sivonen *et al* 1989a

Contd……

Table 2. Contd……

		Microcystis sp.	Codd *et al* 1997
		Oscillatoria spp.	Edwards *et al* 1992, James *et al* 1997a, b
		Planktothrix sp.	Sivonen *et al* 1989a
		Phormidium favosum	Gugger *et al* 2005
Anatoxin-a(s), (AnTx-a(s)	Nerve synapse Antichlorinesterase	*Anabaena flos-aquae, Anabaena lemmermannii*	Onodera *et al* 1997, Matsunaga *et al* 1989 Mahmood and Carmichael 1986 and 1987 Matsungaga *et al* 1989
		Anabaena flos-aquae	Mahmood and Carmichael 1986, Henriksen *et al* 1997, Matsunaga *et al* 1989
		A. lemmermannii	Onodera *et al* 1997
Homoanatoxin-a (HAnTx)	Nerve synapse	*Oscillatoria rubescens*	Aas *et al* 1996
		Phormidium sp.	Wood *et al* 2007
		Planktothrix formosa	Skulberg *et al* 1992
Saxitoxins (STx) (Red-tide algae) (paralytic shellfish poisonings; PSPs)	Nerve axons Sodium channel blocker	*Aphanizomenon flos-aquae,* *Lyngbya wollei* and *Cylindrospermopsis, raciborskii,* *Cylindrospermopsis* and marine dinoflagellates	Sawyer *et al* 1968 Kao and Walker 1982
		Anabaena circinalis	Humpage *et al* 1994, Negri and Jones 1995, Negri *et al* 1997
		Aphanizomenon flos-aquae	Ikawa *et al* 1982, Mahmood and Carmichael 1986
		Cylindrospermopsis raciborskii	Lagos *et al* 1997
		Lyngbya wollei	Carmichael *et al* 1997, Onodera *et al* 1997
Neosaxitoxin (NeoSTx)	Sodium channel blocker	*Aphanizomenon flos-aquae,* *Anabaena circinalis*	Sawyer *et al* 1968, Ikawa *et al* 1982 Humpage *et al* 1994, Negri *et al* 1995
Dermatoxic alkaloids			
Skin irritants			
Aplysiatoxin	Skin, protein kinase C activator	*Lyngbya, Oscillatoria*	Mynderse *et al* 1978, Fujiki *et al* 1990
	Gastrointestinal illness	*Schizothrix calcicola*	Mynderse and Moore 1987
Lyngbayatoxin-a	Skin, gastrointestinal potent tumour promter	*Schizothrix, Oscillatoria, Lyngbya majuscula*	Mynderse *et al* 1977, Cardellina *et al* 1979, Fujiki *et al* 1990, Fujuki *et al* 1984, Aimi *et al* 1990
	Inflamatory agent, severe oral and gastrointestinal inflamatory agent	*Lyngbya majuscula*	Serdula *et al* 1982, Izumi and Moore 1987 Fujiki *et al* 1990
Debromoaplysiatoxin	Skin Inflamatory activator	*Oscillatoria, Schizothrix* and *Oscillatoria nigroviridis*	Mynderse *et al* 1977, Moore *et al* 1984, Fujiki *et al* 1984
Lipopolysaccharides (LPS)	Endotoxin, potential irritant; affects any exposed tissues	*Anacystis nidulans, Schizothrix calcicola,* *Oscillatoria brevis,* *Anabaena flos-aquae, Oscillatoria tenuis,* *M. aeruginosa, Anabaena variabilis*	Ressom *et al* 1994, Weise and Drews 1970
Cyclic guanidine alkaloids			
Cytotoxins	Cell line	*Cylindrospermopsis raciborskii*	Hawkins *et al* 1985, 1997
		Umezakia natans	Harada *et al* 1994
		Aphanizomenon ovalisporum	Banker *et al* 1997

cyanobacterium *Anabaena flos-aquae* NRC 44-1 (Table 2). AnTx-a is a low molecular weight alkaloid (MW=165 daltons, M/Z; $C_{10}H_{15}NO$) and a potent post-synaptic neuromuscular blocker (Carmichael *et al* 1997).

b. Anatoxin-a (s) (AnTx-a(s)) is a phosphate ester of a cyclic N-hydroxy-guanidine methyl (MW=252 daltons M/Z, $C_7H_{17}N_4O_4P$; Fig. 3B). It has been reported in blooms and isolated strain of *Anabaena lemmermannii* (Table 2). When injected into laboratory mice it produces marked salivation.

c. Homoanatoxin-a (HAnTx) is a unique and potent neuromuscular blocking agent and has been reported from *Planktothrix rubescens* and *Phormidium formosa* (FiG. 3C; Table 2).

d. Saxitoxins (STx) is a group of carbamate alkaloid neurotoxins (Fig. 3D). This group of toxins is produced by several cyanobacteria and certain genera of marine dinoflagellates (red-tide algae; Table 2). They are also known as paralytic shellfish poisonings (PSPs; Hallegreaff 1993) in the freshwater mussel *Alathyria condola* (Negri and Jones 1995).

e. Neosaxitoxin (NeoSTx) is similar to STx (Fig. 3E) in molecular weight and found in two species of cyanobacteria and also in a marine dinoflagellate (Table 2). Although STx and NeoSTx occur in some strains of the cyanobacterial genera *Anabaena* and *Aphanizomenon*, they are better known from producers of 'red-tides' (Table 2).

1.4.3. Skin irritants

Some marine cyanobacteria contain dermatotoxic alkaloids (skin irritants) like lyngbyatoxins and aplysiatoxins that are also associated with gastro-enteritis, fever, arrest of respiratory muscles and paralyzing of skeletal and neuromuscular systems. These toxins cause death within few minutes. In mouse bioassay death by respiratory arrest occurs rapidly (within 2-30 min; Sivonen and Jones 1999). Some marine cyanobacteria of the genera *Lyngbya, Oscillatoria* and *Schizothrix* may produce toxins, aplysiatoxins (Fig. 3F), debromoaplysiatoxins (Fig 2G) and lyngbyatoxin (3H). These toxins may cause skin problems in swimmers (Table 2). Lipopolysacchride (Fig. 4) was first isolated from the cyanobacterium *Anacystis nidulans* (Weise and Drews 1970) may and cause allergy in mammals when they come in contact with the compound (Table 2).

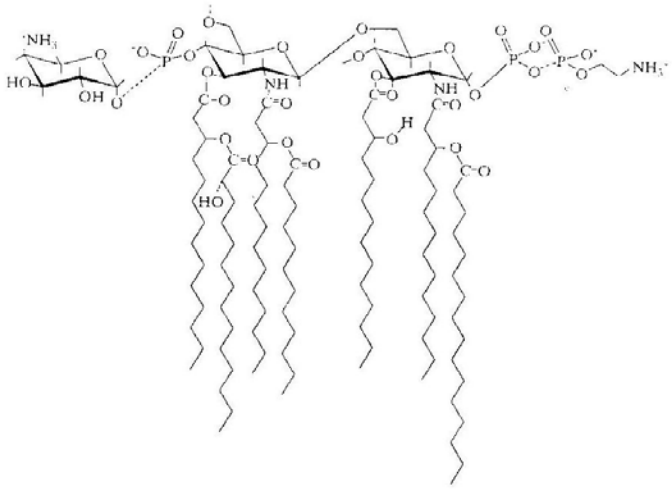


Fig. 4. General Chemical structure of lipopolysaccharide endotoxin (Weise and Drews 1970)

1.5. Isolation and culturing of cyanobacteria in different growth media

The methodology used for isolation, purification and mass culturing of cyanobacteria are quite different as compared to other bacteria. Most cyanobacteria can readily be distinguished from other phytoplanktons under the microscope on the basis of their morphological features. Several studies have been done on the isolation and purification of cyanobacteria (Andersen and Kawachi 2005). In laboratory, culturing of cyanobacteria requires conditions similar to the natural environments. Under favorable conditions, the population can increase dramatically.

The cultivation and growth of cyanobacteria can be difficult, as different strains require specific type of environmental conditions. The requirements of cyanobacteria for vitamins, organic and inorganic constituents (Andersen and Kawachi 2005) vary from species to species. The cyanobacteria species not only require different constituents but also need different conditions/concentrations of pH, salinity, temperature, light, aeration etc. (Guillard and Morton 2003, Andersen and Kawachi 2005).

Several liquid and solid media have been developed by scientists (Andersen and Kawachi 2005) to develop rapid culture for isolation, purification and mass culturing of cyanobacteria. Generally culture media IMR ½, Z8, K, L, ES, ASN III, F/2, BG 11, B-12, CB, MN and SAG 1 are used for culturing of fresh and marine waters species.

1.6. Taxonomy of cyanobacteria

According to Brenner *et al* (2001) the classification of microbes is the arrangement into taxonomic groups (ranks) and it reflects the evolutionary relationships between organisms (Komárek 2003).

There are several methods that have been developed to identify cyanobacterial species like light microcopy, compound, inverted and epifluorescence microscopes and methods comparing DNA-sequences.

In the present study the classification and identification of species (Table 7) are based on classification schemes of Komárek and Anagnostidis (1999, 2005). Recently, Hoffmann *et al.* (2005) proposed classification system of cyanobacteria on the base of genetic relationships, mainly 16S rDNA gene sequences, morphology and thylakoid arrangements. The 16S rDNA gene has a universal distribution in prokaryotes. The phylogenetic analysis of the 16S rDNA gene has revealed close relationships among cyanobacteria and has a central role in inferring phylogenetic relationships and in identification of bacteria.

1.7. DNA sequence analyses

Cyanobacteria occupy diverse habitats in the environment (see 1.1). To understand the ecology and taxonomy of cyanobacteria, it is necessary to know the relationships among different cyanobacterial species in nature. Traditionally microscopy is used to identify strains on the basis of morphological characteristics. However, morphology may change depending on environmental (Evans *et al* 1976) and culturing conditions (Garcia-Pichel *et al* 1996). Microscopic based studies also require time and certain level of experience to determine key morphological characteristics (Scholin *et al* 2003). These days DNA sequence analysis (Roeselers *et al* 2007), is a most reliable method to identify cyanobacteria (Anjos *et al* 2006) up to species level and helpful in order to prepare phylogenetic trees. The phylogenetic analyses are used to estimate the evolutionary relationships among organisms.

DNA sequencing e.g. at the small sub-unit ribosomal DNA (16S rDNA gene; Robertson *et al* 2001, Anjos *et al* 2006) and the *cpc*BA (phycocyanin DNA-region; Neilan *et al* 1997, Janson and Granéli 2002) has been used to inter phylogenetic relationships in cyanobacteria. According to Ludwig and Klenk (2001, 2005) the sequence analyses include alignment of sequences, construction of a phylogenetic tree and testing the reliability of the constructed tree with bootstrapping. ClustalW (Chenna *et al* 2003) is a computer based programme and used for alignment of sequences. The relationships of the aligned sequences are shown in

the form of a phylogenetic tree. The branching of tree displays the inferred evolutionary relationships of the strains and bootstrap values (Nei and Kumar 2000).

1.8. Bioassays

A natural product isolation programme generally involves preliminary toxicity assessment and/or pharmacological evaluation. The primary bioassays can be rapidly applied to screen a large number of samples. A bioassay made it possible to discover new compounds for pharmaceutical, agricultural or biocontrol applications and to assess its toxicity.

Several *in vitro* cytotoxicity assays are available for the detection of cyanobacterial toxicity. In the present study two assays were used to detect the presence of cyanotoxins.

1.8.1. Brine Shrimp (*Artemia franciscana*) Bioassay

Artemia franciscana (brine shrimps) is a species of aquatic crustaceans. They are found in brine lakes. Unhatched brine shrimp cysts are metabolically inactive and can remain in total stasis for several years while kept in cold and dark conditions. Brine shrimp can tolerate varying levels of salinity, and common biology experiment conditions. The nauplii, or larvae, of brine shrimp are used for *Artemia* lethality assay.

Bioactive natural compounds are often toxic to brine shrimp larvae (Feuillade *et al* 1996, Metcalf *et al* 2002, Martins *et al* 2007). Hence *in vivo* lethality to shrimp larvae can be used as a rapid and simple preliminary screening of bioactive compounds during the isolation of natural products.

Cytotoxic screening assay is one of the reliable methods to screen toxicity of cyanobacterial toxins on mammalian cell line. Microcystin-LR and nodularin also have lethal effects on cultures of rat and salmon hepatocytes (Mankiewicz *et al* 2001). Some work has also been reported on cytotoxic assessment of marine cyanobacterial strains belonging to the genera *Synechocystis* and *Synechococcus* isolated from the temperate Atlantic coast of Portugal (Martins *et al* 2008).

1.9. Detection of compounds by liquid chromatography tandem mass spectrometry (LC-MS/MS)

LC-MS is a powerful technique to detect the presence of cyanobacterial toxins especially MC and NODLN in cyanobacteria as mentioned by Rohrlack *et al* (2003), Zhang *et al* (2004), Diehnelt *et al* (2005) and Anjos *et al* (2006). This successful method is also used to

screen variants of MC from blooms and cultures (Anjos *et al* 2006) and other secondary metabolites (Puddick and Prinsep 2008).

1.10. Aims and objectives of the current study

The main aim of this thesis is to provide new knowledge about cyanobacteria and their bioactive compounds from Pakistan and Norwegian collected from marine and brackish waters. The sub goals of this study have been:

1. To isolate some monospecific strains of cyanobacteria from the coastal waters in Norway and Pakistan.
2. Find suitable growth conditions for new strains.
3. To identify and characterize the cyanobacterial species by microscopy and DNA sequencing and determine their systematic positions.
4. Reveal genetic differences within a morphospecies.
5. To determine bioactivity by *Artemia* bioassay of fresh and extracts of freeze dried cyanobacteria collected from Norwegian and Pakistan waters.
6. To determine the presence of potentially bioactive cyanbacterial oligopeptides in cyanobacterial species by using LC-MS/MS technique.
7. Obtain information about chemical nature of bioactive compounds.

2. MATERIALS AND METHODS

2.1 Collection of cyanobacterial strains

a. Norwegian strains

Norwegian strains were collected from the following areas.

1. Samples from sandy shore in Hulvika, outer Oslofjord collected on 20-09-06 by Kjetil Røberg.
2. Samples from sandy shore near Kaupang North Norway collected on 28-08-06 by Kjetil Røberg.
3. Samples were taken from rocks, submerged stones and pebbles from the rocky shore of Huk, Bygdøy, inner Oslofjord.
4. In addition, strains were obtained from culture collection of Norwegian Institute for Water Research (NIVA), University of Oslo (UiO) and University of Bergen (UiB; see Results Table 7).

b. Pakistani strains

Pakistani cyanobacterial strains were isolated from Astola Island, Pasni (25º 03' N and 63º 50' E; rocky shore and open sea), Gawadar (25 º 86' N and 63º 65' E; rocky shore and open sea) and Ormara, (25º 21' N, 64º 61' E, open sea; Table 7) Balochistan in 2006 by scraping of pebbles and rocks and by using phytoplankton net (Becker 1994), respectively. The net (mesh size 55 μm) was towed at the water surface during day light hours. The collected plankton samples were passed through a net of mesh size 60 μm to eliminate the large zooplanktons. The samples were diluted with filtered seawater and immediately brought to the laboratory.

2.2. Preparation of growth media

For culturing of cyanobacteria different media IMR ½ (30 and 16 PSU; Epply *et al* 1967 modified by Paasche 1971), ES (30 and 16 PSU; Provasoli 1968), Z8 (16 PSU Staub 1961 modified by Kotai 1972) and ASN III (34 PSU; Rippka 1988) were used to obtain good growth (Appendix 1). For Norwegian strains seawater was used from Drøbak from 30-40 m depth. The water was filtered through GF/C (Whatmann glassfibre filters). For Pakistani strains the seawater was used from Sandspit, Karachi and filtered through an ordinary filter paper.

2.2.1. Isolation and methods

In this study the following methods for isolation and purification were used to obtain a single clone or colony (Fig. 5).

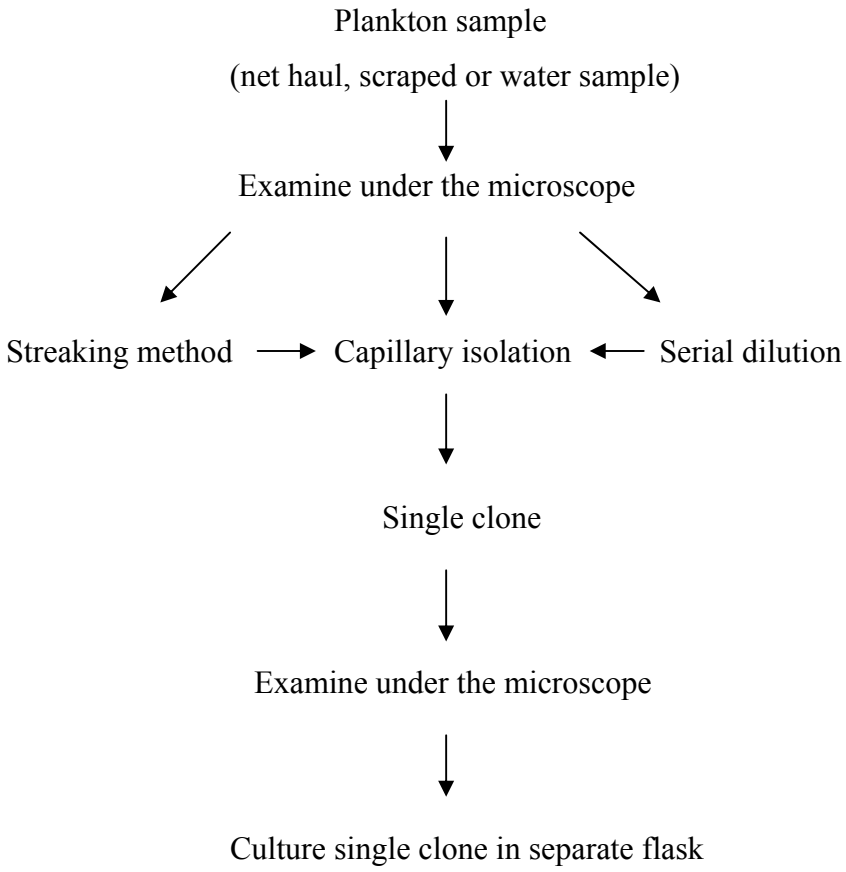


Fig. 5. A method to obtain a single cell or colony from natural sample of micro algae *(with the curtsey of Bente Edvardsen).*

a. Streaking and capillary methods

The benthic cyanobacteria were isolated by streaking method (Hoshaw and Rosowski 1973) on solidified agar plates. In a flask 8 g agar was dissolved in 300 mL distilled water while in another flask 700 mL of medium (IMR ½ for Norwegian strains and ASN III for Pakistani strains) was autoclaved at 15 lb of pressure at 122 ºC for 20 min. After cooling both solutions were mixed. The luke warm medium was poured over petri plates and allowed to set at 15 ºC.

After solidification of agar cyanobacterial sample was streaked over solidified medium and the petri plates were kept under continuous white cool illumination and in suitable temperature in order to obtain best growth of cyanobacterial species. The single cell or filament of cyanobacteria was picked by capillary method (Andersen and Kawachi 2005). After getting single cell or filament, it transferred into Nunclon dish well containing 1 mL algal suitable growth medium (Table 7).

b. Serial dilution culture (SDC) method

Serial dilution method is used to isolate planktonic cyanobacteria as described by Guillard (1973) modified by Andersen and Throndsen (2003). For serial dilution 5 test tubes containing growth medium (Table 7) were used. Serial dilution was done by transferring 1 ml into first tube, second tube to last tube. The dilutions were 10x, 100x, 1000x, 10000x and 100000x. The tubes were kept under continuous illumination at different temperature culture rooms for 4-5 weeks (Table 7).

2.3. Culturing and maintenance of cyanobacterial strains

Purified cyanobacterial cells/colonies were inoculated into 500 mL flasks containing 300 mL of growth medium (Appendix 1) with respect to nutrient requirements of different cyanobacterial species (Table 7). The flasks were kept under constant illumination of cool white fluorescent light and the cyanobacteria were allowed to grow photoautotrophically. In 15 days, a sufficient growth of cyanobacteria was obtained. Two fifty mL of fresh culture was filtered onto a GF/F filter for LC-M/MS analyses and 50 mL filtered onto a polycarbonate filter paper (8.0 μm pore size) for DNA analyses. Fresh medium was added in the flasks for further culturing and microscopic analyses.

To maintain cyanobacterial strains the growth medium was regularly changed on monthly basis. One mL of old culture was transferred into 75 mL of growth media. All cultures were kept in different temperature controlled culture rooms under continuous illumination. Light intensity was measured with a model QSL-100 photometer (Biospherical Instrument Inc., USA). To find the best growth medium, different media were tested. The density of cyanobacterial species were observed by naked eye.

2.4. Chemical structures

The chemical structures of all cyanotoxins were drawn by using ACD/ChemSketch (http://www.acdlabs.com/products/chem_dsn_lab/chemsketch/).

2.5. Identification and morphological characterization

Most cyanobacteria can readily be distinguished from other phytoplankton algae and particles under the microscope on the basis of their morphological features i.e. size, cell structure, shape and filamentous and non-filamentous morphology.

For identification of strains small drop of fresh culture was spreaded on a glass slide and covered with a glass cover slip. A drop of immersion oil was placed over the cover slip and the slide observed at 100x magnification under dissecting microscope lighting system (DMLS; Leica, Germany). The measurements were done in µm by using a computer based program (available with microscope) and pictures taken by a digital sight camera (DS-SM, Japan). For unicellular strains 30 cells were measured and the length and width were noted. For filamentous micro-algae 30 cells were measured and the length and width of filament and coils were also noted. The strains were indentified on the bases of their morphological characteristics. The literatures used for identification were: Desikachary (1959), Komárek and Anagnostidis (1999, 2005) and John *et al* (2002).

2.6. DNA extraction

To target partial phycocyanin DNA-region (*cpc*BA) and partial 16S rDNA-region (SSU; Appendix 4) the DNA was extracted by using Easy Nucleic Acid Isolation (E.N.Z.A.®) SP Plant miniprep Kit (Omega Bio-tek, USA). Two varieties of the method were used for isolation of DNA from cyanobacterial samples, the first for fresh and the other for freeze dried material. To know the amount of DNA in PCR products Nanodrop method was used (ND-1000 spectrophotometer at 230 λ).

2.6.1. For freeze dried and powdered Pakistani samples

Twenty mg of powdered cyanobacterial strains from Pakistan were weighed. Six hundred µl Buffer SP1 was added into powdered cyanobacterial strain followed by addition of 5µl RNase solution in an Eppendrof tube. The solution was thoroughly mixed by vortexing to obtain better extraction of DNA and incubated at 65 ºC on block heater (Techne, DRI-Block, DB.2A, UK) for 10 min. After incubation 210 µl Buffer SP2 was added.

2.6.2. For fresh Norwegian samples

Fifty mL of fresh culture was filtered through a polycarbonate filter. Four hundred µl Buffer SP1 was added into Eppendorf containing the polycarbonate filter. The sample incubated at

65 ºC for 10 min. The filter was taken and then removed. After incubation 140 µl Buffer SP2 was added.

The following procedure was the same for both fresh and freeze dried samples.

The sample was incubated for 5 min on ice and centrifuged at 13000 rpm (Eppendorf, 5415 D, Germany) for 10 min. The supernatant aspirated onto an Omega Homogenizer Column, then centrifuged the sample at 13000 rpm for 2 min to remove the remaining precipitates and cell debris. The clear lysate was transferred into a new 1.5 µl collection tube. I measured the volume of the lysate for the next step (750 µl of SP3).

Six hundred fifty µl supernatant was transferred to a HiBind® DNA column and I centrifuged the column at 13000 rpm for 1 min to bind DNA (repeated 2 times). The column was placed in a new 2 mL collection tube added 650 µl SPW Buffer and centrifuged the sample at 13000 rpm for 1 min.

The column was again transferred to a new Eppendorf tube and added 60 µl Elution Buffer (pre-warmed at 65 ºC) and incubated at room temperature for 3-5 min. The sample was centrifuged at 13000 rpm for 1 min to elute DNA. I repeated the same elution procedure by adding 40 µl of Elution Buffer. The extracted DNA was stored at -20±2 ºC until later use.

a. PCR amplification

For PCR all chemicals (PCR water, PCR Buffer, dNTP, primers, and DNA template) were thawed on ice for 30 min, except Taq Polymerase and Taq Master Enhancer (TME; Mater Taq Kit, Eppendorf, Germany). I added PCR water, PCR Buffer (5 µl; pre-warmed at 65 ºC), Taq Master Enhancer (5 µl), dNTP (2.5 µl), forward and reverse primers (0.5 µl), Taq polymerase (0.1 µl) and DNA template (5 µl) in a mix reaction tube (Nübel *et al* 1997 and Robertson *et al* 2001). Taq polymerase was added at last. The PCR tubes were loaded with 45 µl of mix and 5 µl DNA template and the PCR tubes were incubated in thermo Mastercycler (Eppendorf, Germany). For amplification of the 16S rDNA and phycocyanin genes, different sets of primers were used (Table 3; Appendix 4) in the reaction with an amplification profile consisting of a denaturation of DNA at 94 ºC for 5 min followed by DNA synthesis and denaturation at 94 ºC for 1 min 35 cycles, primerannealing for 1 min 35 cycles at 50, 55 and 60 °C for 16S rDNA and 45 and 50 °C for phycocyanin (Appendix 4) and an extension at 72 ºC for 1 min 35 cycles. This was followed by a final elongation step of 72 ºC for 10 min.

Table 3. Different primer sequences and their target sites used in the present study. (F-forward and R-reverse).

Primers	Sequences (5' to 3')	References
For 16S rRNA		
CYA106F	CGG ACG GGT GAG TAA CGC GTG A	Nubel *et al* 1997
CYA781R(a)	GAC TAC TGG GGT ATC TAA TCC CAT T	Nubel *et al* 1997
CYA359F	GGG GAA TYT TCC GCA ATG GG	Nubel *et al* 1997
CYA781R(b)	GAC TAC AGG GGT ATC TAA TCC CTT T	Nubel *et al* 1997
For phycocyanin		
PCαR	CCA GTA CCA CCA GCA ACT AA	Neilan *et al* 1995
PCβF	GGC TGC TTG TTT ACG CGA CA	Neilan *et al* 1995
cpcBF	TAG TGT AAA ACG ACG GCC AGT TG (C/T) (C/T) T (G/T) CGC GAC ATG GA	Robertson *et al* 2001
cpcAR	TAG CAG GAA ACA GCT ATG ACG TGG TGT A(G/A)G GGA A(T/C)T T	Robertson *et al* 2001

b. Electrophoresis

Agarose (0.4 g; Merck, Germany) was dissolved in 50 mL TAE buffer solution and melted for 1 min to make 0.8% agarose gel. When the temperature of agarose became 50 ºC approximately, I added one drop of etidium bromide (EtBr; 0.6325 mg/mL; Gene Chrom, USA) and poured the agarose gel into an electrophoresis plate. After 30 minutes I transferred the gel into the electrophoresis vessel (Bio Rad, wide mini sub^{TM} cell, Italy) and filled with 1x TAE buffer solution. One µl loading buffer solution (GBL; Eppendorf, Germany) was mixed with 5 µl PCR product and loaded to the slots in the agarose gel, while in the first slot 2 µl DNA size marker (Eco RI/Hind III, preheated to 65 ºC; Eppendorf, Germany) was loaded. I ran the gel 80 V (Electrophoreses power supply, EPS-301; Amersham Pharmica Biotech, USA) for 30 min and observed the bands by using gel documentation chamber (SynGene, UK).

c. Cleaning of PCR products for DNA analyses

For cleaning and purification of DNA (Wizard® SV PCR Clean-Up System, USA) an equal volume of membrane binding solution (30 µl) and PCR product (30 µl) were used and incubated for 1 min at room temperature. The tubes were centrifuged at 13000 rpm for 1 min. Seven hundred µl membrane wash solution (ethanol included) was used for washing of DNA. I then centrifuged the tubes at 13000 rpm for 1 min and repeated washing with 500 µl

membrane washing solution. For elution of DNA 50 µl nuclease-free water was used. The DNA was incubated at room temperature for 1 min. I centrifuged the tubes at 13000 rpm for 1 min and stored the samples at -20 ºC.

The samples were prepared for DNA sequencing by adding 9 mL of PCR water, 1 µl PCR product and 2 µl respective forward primer. In another eppendorf tube 9 µl of PCR water, 1 µl PCR product with 2µl reverse primer were added. The samples were delivered to the ABI lab, University of Oslo for sequencing using the BigDye® Terminator v3.1Cycle Sequencing Kit (3730 DNA analyzer, USA).

d. Analysis of DNA sequences and construction of phylogenetic trees

The DNA sequences were analyzed by BioEdit. v7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) provided by National Centre of Biotechnology Information ((http://www.ncbinlm.nih.gov). The chromatograms of DNA sequenced strains were opened in BioEdit. The messy sequences, in the beginning and in the end of chromatograms, were deleted to obtain good results. The forward and reverse sequences were placed in an order, that the first covered the first part and the last the last part of the consensus sequence. All consensus sequences were checked base for base and corrected manually. The corrected sequences were analyzed on BLAST (blastn; http://www.ncbi.nlm.nih.gov/BLAST) provided by GenBank molecular database of National Centre for Biotechnology for Information (NCBI; http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html). The DNA sequences obtained were compared with available sequences in GenBank by BLAST search. As a rule 2 most similar sequences were downloaded and included in the alignment and phylogenetic analyses. For automatic multiple aligning of the whole sequence matrix I used a computer based programme ClustalW (http://www.es.embnet.org./Services/production.html). After ClustalW and manual adjustments of sequences the alignments submitted for phylogenetic analysis (http://www.paup.csit.fsu.edu/index.html) on MEGA (Beta v4.1). I selected bootstrap test of phylogeny and chose the neighbor-joining and minimum evolution, which gave the best phylogenetic trees.

2.7. Bioassays

2.7.1. *Artemia franciscana* (brine shrimp) assay

Pakistani strains were harvested through centrifugation (3000 rpm for 10 minutes at 10 ºC; Hitachi, Japan) and freeze dried (Tiro, Japan) at Hussain Ebrahim Jamal Research Institute of Chemistry (HEJRIC), University of Karachi, Pakistan and stored at -20 °C.

To know the concentration of cells in culturing media, 2 ml of fresh culture of Norwegian strains were filtered through GF/F paper (Whatman) and dried overnight in an oven (Eppendorf, Germany) at 50 °C. For dried Pakistani strains 2 mg of freeze dried cells were dried overnight at 50 °C (Table 4).

Table. 4. Dry weights of *Geitlerinema* sp. UK-G-106 and freshwater positive controls to calculate the concentration of strains used in *Artemia* assay.

Strains	Code nos.	Weight of filter (a) gm	Amount of strains	Dried weight with filter (b) gms	Total dry weight (c) gms (a-b=c)
Geitlerinema sp.	UK-G-106	0.037	2 mg	0.103	0.0659
Planktothrix agardhii	NIVA-CYA 229	0.038	2 ml	0.038	0.0004
P. rubescens	NIVA-CYA 407	0.038	2 ml	0.038	0.0004
Microcystis aeruginosa	NIVA-CYA 166	0.037	2 ml	0.037	0.0001

For *Artemia* assay fresh cultures of Norwegian strains and methanol extracts of Pakistani strains were used. Two mg of dried cells of Pakistani cyanobacterial strains were extracted in 2 mL of MeOH:H_2O (50:50; v/v) solution for 1h and centrifuged for 5 min at 16000 rpm. The supernatants were evaporated in a rotary evaporator (Speed Vac Concentrator, Savant) at 0 ºC for 3 h. The dried extracts were obtained and dissolved in 6 mL of 100% autoclaved seawater for further application.

Hundred mg of cysts of *Artemia franciscana* (Creasel, Deinze, Belgium) were incubated for hatching in 250 mL of 70% filtered (Whatman GF/C) and autoclaved seawater for one day at 25 ºC in light (150 µmol m^{-2} s^{-1}) and aerated with an aquarium pump. After two days the second stage of nauplii were obtained. Ten nauplii were counted and transferred into each of 24 wells of Nunclon multiwell dish. I made a serial dilution of alive Norwegian and methanolic extracts of Pakistani strains (1x 10x, 100x and 1000x at the concentrations of 1, 0.1, 0.01, 0.001 mg dw mL^{-1}). Only one Pakistani strain UK-G-106 was diluted (10x, 50x, 100x, 300x, 500x and 600x at the concentrations of 0.1, 0.02, 0.01, 0.003, 0.002, 0.0016 mg dw mL^{-1}) by transferring 1 mL into 5 test tubes with seawater. One mL of each dilution was transferred into Nunclon multiwell dish. Four replicates were used for each dilution.

Seawater and sodium dodecyl sulfate (SDS) were used as negative and positive controls (n= 40 *Artemia* nauplii), respectively (Table 5).

In one experiment three species of freshwater toxic cyanobacteria were used as positive controls i.e. *P. rubescens* NIVA-CYA 407 and *Planktothrix agardhii* NIVA-CYA 229 the dilutions were 2x, 0.2x, 0.02x, 0.002x (at the concentrations of 1, 0.1, 0.01, 0.001 mg dw mL^{-1}). For another positive control *Microcystis aeruginosa* NIVA-CYA 166 the dilutions were 0.5x, 0.05x, 0.005x, 0.0005x (at the concentrations of 2, 0.2, 0.02, 0.002 mg dw mL^{-1}). The vials were incubated in the dark at room temperature for 24 h. After this the number of dead and survived nauplii was recorded. The data were analyzed and LC_{50}-24 h was determined.

Table 5. Concentration of sodium dodecyl sulfate (SDS; positive control).

SDS (mg/L)	**Volume SDS** (0.01%) mL	**Volume** (70% seawater mL)
10	1	9
13.5	1.35	8.65
18	1.8	8.2
24	2.4	7.6
32	3.2	6.8

2.7.1.1. Fractionation of cyanobacterial strain UK-G-106 with C_{18} column

a. Extraction of cyanobacterial material

I weighed 50 mg of cyanobacterial strain UK-G-106 and extracted in 10 mL MeOH:H_2O (50:50, v/v) at room temperature for 1 h. The extract was centrifuged at 4 ºC and 16000 rpm. The supernatant was transferred into a new Eppendorf tube, dried in a Vac speed concentrator (Savant) for 6 h and kept at -20 ºC.

b. Pre-conditioning of C_{18} column

Ten mL of 100% MeOH was added in a 10 mL syringe (BD Plastipak, Spain) and passed through pre-packed C_{18} cartridge/column (Sep-Pak®, UK; 1 mL/min) solid phase extraction (SPE). Ten mL dist. H_2O (1 mL/min) was passed through the column to get rid of MeOH.

c . Fractionation of dried extract (MeOH:H_2O; 50:50) of cyanobacterium UK-G-106

Ten 10 mL of dist. H_2O (0% MeOH; the 1st extract) was added to freeze dried extract of UK-G-106 and passed through the C_{18} column slowly (1 mL/min). Then similarly 10 mL 20% MeOH (2nd extract), 40% MeOH (3rd extract), 60% MeOH (4th extract), 80% MeOH (5th extract) and 100% MeOH (6th extract) were passed through column to elute fractions at different polarities (repeated twice). The elute was dispensed in 6 different Eppendrof tubes to evaporate the liquid at 4 ºC for 6 h. I used 4 tubes for the *Artemia* assay, 4 parallel wells and 6 different concentrations.

2.8. Isolation and purification of oligopeptides by liquid chromatography tandem mass spectrometry (LC-MS/MS)

This part was performed by Thomas Rohrlack at NIVA.

Oligopeptides were extracted from filters with cultured Norwegian and powdered Pakistani cyanobacterial strains. After lyophilisation 50% MeOH was used as described previously (Rohrlack *et al* 2003). For the detection and identification of oligopeptides, Liquid Chromatography Mass Spectroscopy (LC-MS/MS) was used. The instrumental setup included a Waters Acquity Ultra-performance Liquid Chromatography (UPLC) System equipped with a Waters Atlantis C_{18} column (2.1 x 150 mm, 5 µm particle size) and directly coupled to a Waters Quattro Premier XE tandem quadrupole MS/MS detector. The UPLC system was set to deliver a linear gradient from 10% to 45% acetonitrile in water, both containing 0.1% formic acid, within 10 minutes at a flow rate of 0.25 mL min^{-1}. The column and auto sampler temperatures were 20 and 4ºC, respectively. At all times, the MS/MS detector was run in positive electrospray mode (ESI+). Other general settings included a source temperature of 120 ºC, a desolvation temperature of 350 ºC, a drying gas flow rate of 800 L h^{-1}, a gas flow at the cone of 50 L h^{-1}, and standard voltages and energies suggested by the manufacturer for the ESI+ mode. Only the cone voltage and the settings for the collision cell were adapted to the various types of analysis. Nitrogen, continuously delivered by a nitrogen generator (NG 11, Parker Balston, USA), served as drying, nebulising, and cone gas.

To screen extracts for cyanobacterial oligopeptides, the detector was run in total scanning mode for the mass range of 500 to 2000 Da during the entire UPLC gradient. At this stage, the cone voltage was 50 V and the time for one scan 2 seconds. Afterwards, all mass signals, that represented compounds with a molecular mass within the range of 500-2000 Da, were

analyzed in fragmentation experiments. To this end, the detector was ran in daughter ion scanning mode and the cone voltage and collision cell settings were optimized to obtain as many fragments of the respective compound as possible. In all cases, argon served as collision gas. For the identification of a given compound, its fragmentation spectrum was screened for ammonium ions of amino acids and other analytical fragments to be expected when working with peptides. Larger fragments were identified by comparison with fragment patterns of already elucidated compounds or available standard material. Further information was gained from using the fragmentation simulation software HighChemMass Frontier (version 3). Finally, identified fragments, the molecular mass of the respective compound, its isotope spectrum and fragmentation patterns of already elucidated oligopeptides were used to gradually develop a putative model of the compound's plain structure, which then was compared to structures of oligopeptides already described in the literature (Welker and von Döhren 2006). This way of structural elucidation of cyanobacterial oligopeptides on the basis of MS fragmentation experiments has been successfully used in several earlier studies (Batemann *et al* 1995, Fastner *et al* 2001, Welker *et al* 2004a, b, Tooming-Klunderud *et al* 2007).

3. RESULTS

3.1. Isolation of cyanobacterial strains

By streaking and capillary methods 8 benthic filamentous strains *Phormidium* spp. UIO 145, UIO 146, *Geitlerinema* sp., UK-G-106, *Chlorogleopsis* sp. UK-O-105, *Oscillatoria* spp. UK-G-108,UK-G-110, *Pseudoanabaena* spp. UK-O-109 and UK-O-101 were isolated. By dilution method coccoids *Synechococcus* sp. UK-G-102 and *Chroococcus* sp., UK-G-103 were isolated. The capillary method was found to be effective to obtain strains of certain filamentous *Phormidium* spp. UIO 018, UIO 145 and UIO 146 and *Oscillatoria* sp. UIO 017.

3.2. Cyanobacterial strains and growth conditions

In this work 24 strains isolated from brackish and marine waters of Norway and Pakistan, were studied. Of these 12 were isolated in this study. To find a good growth medium for culturing of cyanobacteria, different media were tested (Table 6). Generally the results showed that the ES (30 PSU), IMR ½ (30 PSU) and Z8 (16 PSU) were the best media for culturing the cyanobacteria (strains of Chroococcales and Oscillatoriales isolated from Norway) in this study. It was noted that the Norwegian strains *Synechococcus* spp. UIO 012 and NIVA-CYA 328 could grow in all tested media and grew well in IMR ½ (30 PSU) and Z8 (16 PSU) media, respectively. Pakistani strains were grown in high salinity medium ASN III (34 PSU; Table 7). The Norwegian and Pakistani strains were grown at the light ranging from 25-30 µmol photons $m^{-2}s^{-1}$. However, 2 Norwegian strains, *Synechococcus* spp. UIO 015 and UIO 016 were grown under somewhat lower illumination (12-15 µmol photons $m^{-2}s^{-1}$). The Table 7 represents the collection sites, different growth conditions and other details of studied cyanobacteria.

Table 6. Growth tested in different media (+++ best, ++ good, + low, – no growth).

Taxa	Strain code	Growth conditions				
		IMR 1/2		ES		Z8
		(30 PSU)	(16 PSU)	(30 PSU)	(16 PSU)	(16 PSU)
Chroococcales						
Synechococcus sp.	NIVA-CYA 328	++	+	++	+	+++
Synecococcus sp.	UIO 013	++	-	-	-	-
Synechococcus sp.	UIO 012	+++	+	+	+	+++
Synecococcus sp.	UIO 016	+++	-	++	-	-
Synechococcus sp.	UIO 015	+++	-	++	-	-
Chroococcus submarinus	NIVA-CYA 331	-	+	+	-	++
Chroococcus submarinus	NIVA-CYA 329	++	-	-	-	+++
Oscillatoriales						
Peudoanabaena sp.	NIVA-CYA 333	-	-	-	+	++
Peudoanabaena sp.	NIVA-CYA 280	+	-	+	+	++
Spirulina subsalsa	NIVA-CYA 163	-	-	-	+	++
Spirulina subsalsa	NIVA-CYA 164	-	-	-	+	++
Phormidium sp.	UIO 018	+++	-	+	-	-
Phormidium sp.	UIO 145	+++	-	++	-	-
Phormidium sp.	UIO 146	+++	-	++	-	-
Oscillatoria sp.	UIO 017	+++	-	++	-	-
Oscillatoria cf. *chalybea*	NIVA-CYA 165	-	-	-	+	++

Table 7. Collection sites and culturing conditions of cyanobacterial species in this study.

Systematic position	Taxa	Strain codes	Collection sites	Isolated by	Growth medium	Salinity of medium (PSU)	Growth temperatures (ºC)
Chroococcales			**NORWAY**				
	Synechococcus sp.	NIVA-CYA 328	Holmestrandfjorden, 1997	Skulberg R	Z8	16	16±1
	Synechococcus sp.	UIO 013 syn. Bl.gr	Skagerrak	Wenche E	IMR 1/2	30	16±1
	Synechococcus sp.	UIO 012 syn. sj. gr.	Oslofjord	Throndsen J	IMR 1/2	30	19±1
	Synechococcus sp.	UIO 016 syn. UiB-34	Raunefjorden, 2004	Larsen A	IMR 1/2	30	10±1
	Synechococcus sp.	UIO 015 syn. UiB-15	Raunefjorden, 2004	Larsen A	IMR 1/2	30	06±1
	Chroococcus submarinus	NIVA-CYA 331	Holmestrandfjorden, 1995	Skulberg R	Z8	16	16±1
	Chroococcus submarinus	NIVA-CYA 329	Holmestrandfjorden, 1995	Skulberg R	Z8	16	16±1
Oscillatoriales							
	Peudoanabaena sp.	NIVA-CYA 333	Holmestrandfjorden, 1995	Skulberg R	Z8	16	16±1
	Peudoanabaena sp.	NIVA-CYA 280	Framvaren, 1988	Skulberg R	Z8	16	16±1
	Spirulina subsalsa	NIVA-CYA 163	Oslofjord, 1985	Skulberg R	Z8	16	16±1
	Spirulina subsalsa	NIVA-CYA 164	Oslofjord, 1985	Skulberg R	Z8	16	16±1
	Phormidium sp.	UIO 018 syn. UiO-G	Huk, Oslofjord, 2007	Hameed S	IMR 1/2	30	10±1
	Phormidium sp.	UIO 145 syn. Hulvika	Oslofjord, Hulvika, 2006	Hameed S	IMR 1/2	30	19±1
	Phormidium sp.	UIO 146 syn. Kaupang	Northland, Kaupang, 2006	Hameed S	IMR 1/2	30	19±1
	Oscillatoria sp.	UIO 017 syn. UiO-R	Huk, Oslofjord, 2007	Hameed S	IMR 1/2	30	10±1
	Oscillatoria cf. *chalybea*	NIVA-CYA 165	Oslofjord, 1985	Skulberg R	Z8	16	16±1
Chroococcales			**PAKISTAN**				
	Synechocystis sp.	UK-G-102	Gawadar, 2006	Hameed S	ASN III	34	31±2
	Chroococcus sp.	UK-G-103	Gawadar, 2006	Hameed S	ASN III	34	31±2
Oscillatoriales							
	Pseudoanabaena sp.	UK-O-109	Ormara, 2006	Hameed S	ASN III	34	31±2
	Pseudoanabaena sp.	UK-O-101	Ormara, 2006	Hameed S	ASN III	34	31±2
	Geitlerinema sp.	UK-G-106	Astola Island, Pasni, 2006	Hameed S	ASN III	34	31±2
	Chlorogleopsis sp.	UK-O-105	Ormara, 2006	Hameed S	ASN III	34	31±2
	Oscillatoria sp.	UK-G-110	Gawadar, 2006	Hameed S	ASN III	34	31±2
	Oscillatoria sp.	UK-G-108	Gawadar, 2006	Hameed S	ASN III	34	31±2

3.3. Identification of cyanobacterial strains

In this study a total of 24 cyanobacterial strains (16 from Norway and 8 from Pakistan) were studied, which belong to two orders i.e. Chroococcales and Oscillatoriales. These strains were identified by traditional microscopy on the basis of their cell size and form, cell structures, mucilaginous envelopes and colonial characteristics. The measurements of all strains are shown in Appendices 2 and 3.

3.4. Description of cyanobacterial strains

3.4.1. Norwegian strains

Chroococcales

The identification was based on Komárek and Anagnostidis (1999).

***Synechococcus* sp. Nageli NIVA-CYA 328.** The cells were solitary, free-floating, short rod-shaped, straight or sometimes slightly sigmoid, 0.5-1.3 x 0.5-1.9 µm in diameter and without mucilaginous sheath (Plate 1A). The cells had more or less homogenous and pale blue-green contents. The cells were planktonic in growth medium.

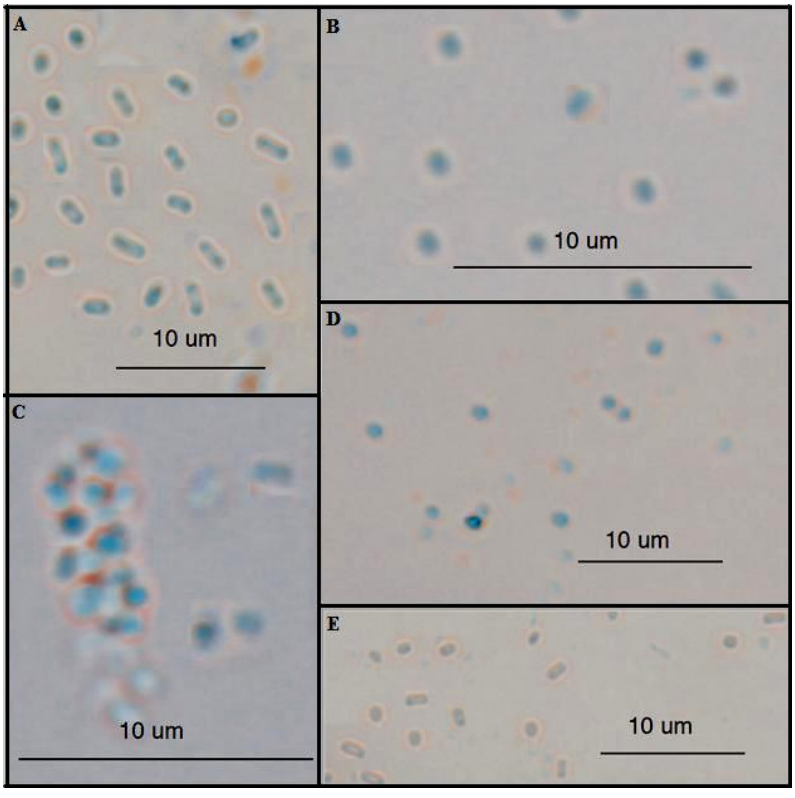


Plate 1. Light micrographs of *Synechococcus* strains used in the present study.
A) *Synechococcus* sp. NIVA-CYA 328
B) *Synechococcus* sp. UIO 013
C) *Synechococcus* sp. UIO 012
D) *Synechococcus* sp. UIO 015
E) *Synechococcus* sp. UIO 016

***Synechococcus* sp. Nageli UIO 013.** The cells were bright green, solitary or grouped in microscopic or macroscopic irregular clusters. The cells were with very fine, colorless, homogenous, mucilaginous sheath. They were long oval or cylindrical or rounded straight, sigmoid (Plate 1B), 0.3 µm up to 0.9 µm wide and 0.4-2.1 µm long. The cells were planktonic in growth medium.

***Synechococcus* sp. Nageli UIO 012.** The cells were bright green, solitary, free-floating, short and rod shaped, without mucilage (Plate 1C). The cell contents were pale blue-green, straight or sometimes slightly sigmoid, 0.1-0.8 x 0.4-1.5 µm. The cells were planktonic in growth medium.

***Synechococcus* sp. Nageli UIO 015.** The cells were bright red, solitary, rarely 2-3 together in rows, more or less cylindrical (Plate 1D) with rounded ends, straight or slightly curved, 0.3-0.8 x 0.3-0.9 µm. Some cells showed solitary granules at poles. The cells were planktonic in growth medium.

***Synechococcus* sp. Nageli UIO 016.** The cells were red, solitary, rarely 3-4 together in rows, more or less cylindrical, straight or slightly curved (Plate 1E), 0.3-1.0 x 0.5-1.2 µm. The cells showed solitary granules at poles. The cells were planktonic in growth medium.

***Chroococcus submarinus* (Hansgirg) Kováčik NIVA-CYA 331**. *Chroococcus* spp. can be distinguished by a dividing pattern in a plane (Plate 2). The cells of *Chroococcus submarinus* are free living or epiphytic. The cells were found in groups and surrounded by mucilaginous sheath. Few cells were found solitary and 16.5-24.5x17.0-24.6 µm in diameter. They formed microscopic, irregular or more or less spherical, gelatinous colonies. The multicellular colonies were packed in packet-like, 2-8-celled groups (Plate 2). Mucilaginous covering was colorless or yellowish. Cells were at first sub-spherical, later hemispherical or in the form of a segment of the sphere. The cells contained blue-green homogenous granules.

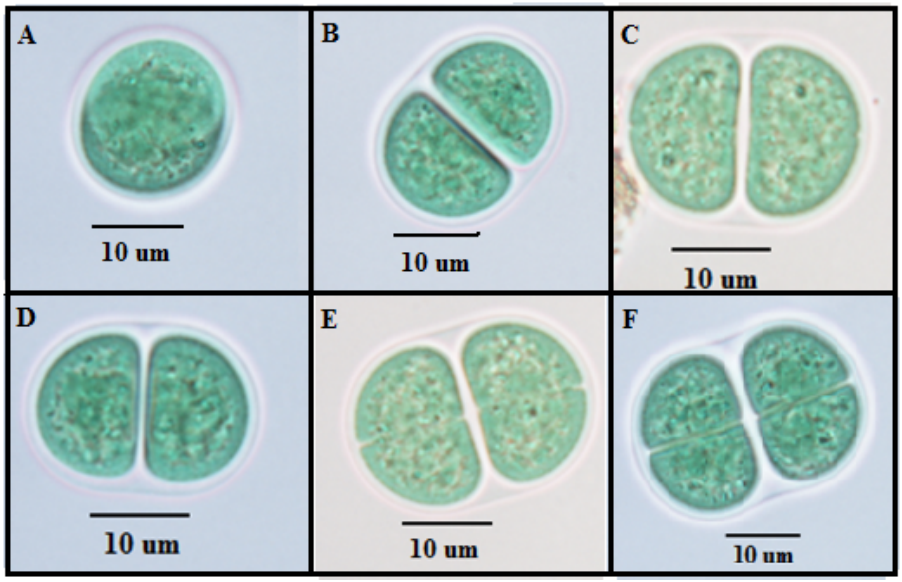


Plate 2. Light micrographs of different stages of cell division in *Chroococcus submarinus* NIVA-CYA 331.

***Chroococcus submarinus* (Hansgirg) Kováčik NIVA-CYA 329.** The cells were green to olive green in color and covered with a mucilaginous sheath. The shape of cells varied from spherical, subspherical, hemi-spherical or irregular-rounded forms; usually many celled (Plate 3), 16.1-25.1x16.3-26.0 µm in diameter. *Chroococcus submarinus* form macroscopic colonies. The colonies were covered by a fine mucilaginous sheath. The cells were planktonic in stagnant medium. The cells divided by binary fission (Plate 3).

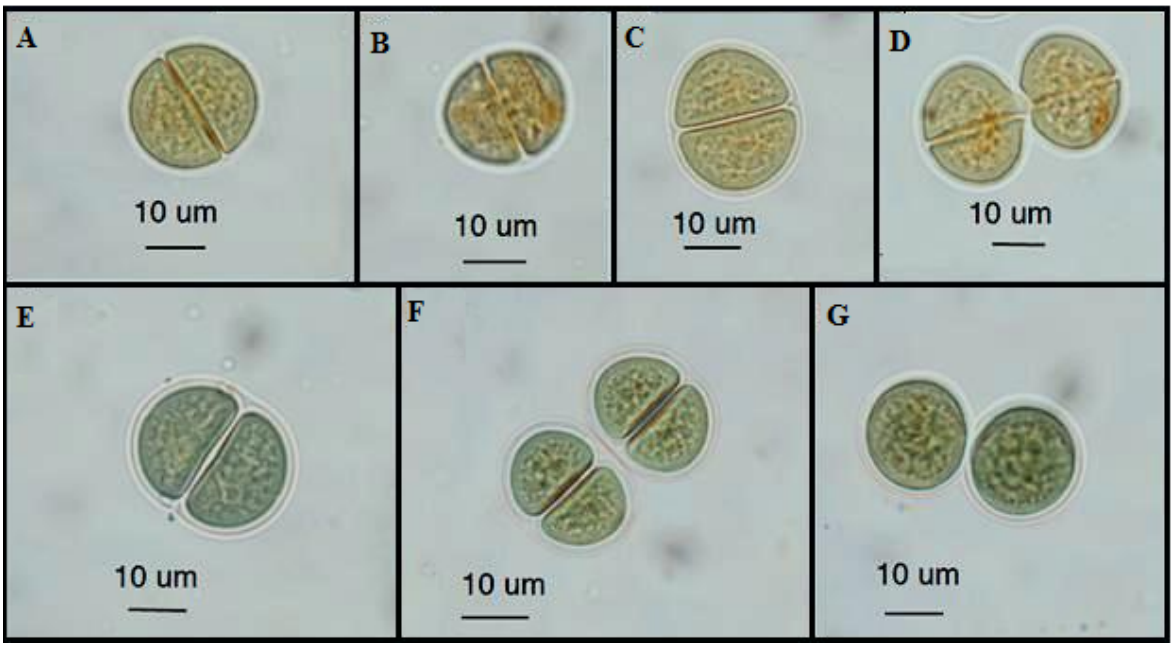


Plate 3. Light micrographs of different stages and cell division in *Chroococcus submarinus* NIVA- CYA 329.

Oscillatoriales

The identification was based on John *et al* (2002) and Komárek and Anagnostidis (2005).

***Pseudoanabaena* sp. Lauterborn NIVA-CYA 333.** Trichomes were solitary in plankton and formed fine mats, cylindrical, ± short, consisting of very few to several cells. Trichomes were without thin and firm sheaths, but often with wide, fine diffluent mucilaginous envelops (Plate 4A). Apical cells were not differentiated, without calyptra and thickened outer cell wall. Motility was lacking. The cells were usually cylindrical with rounded ends, barrel-shaped, longer than wide, 0.8-2.6 wide, 1.8-3.9 µm long and occurred in single cells. Trichomes were benthic in lab cultures and formed sheaths.

***Pseudoanabaena* sp. Lauterborn NIVA-CYA 280.** Trichomes were solitary, straight or slightly curved, motile, strongly constructed, occasionally with thin, indistinct mucilaginous envelops or sheaths. Cells were long-cylindrical, pale-blue green (Plate 4B), 0.1-1.1 µm wide, 1.6-3.0 µm long. Apical cells were rounded. Trichomes were benthic in lab cultures and formed sheaths.

***Spirulina subsala* Oersted ex Gomont NIVA-CYA 163.** Thallus was soft, thin, expanded and mucilaginous. Trichomes were long, bright blue-green in color, regularly densely screw-like coiled (Plate 4C). Coils were tightly joined to one another and arranged parallel. The width of trichomes and coils was 3.1-4.1 µm and 3.2-3.9 µm, respectively. The trichomes were rapidly motile by screw-like or gliding rotation. Apical cells were rounded. In lab cultures they were found attached to the bottom of flask in the form of sheaths.

***Spirulina subsalsa* Oersted ex Gomont NIVA-CYA 164.** Thallus was attached to the flask. Trichomes were pale rose-pink, screw-like coiled, joined one to another, tightly curved (Plate 4D), 3.0-4.1 µm wide, 3.2-4.3 µm high. The trichomes were rapidly motile by screw-like or gliding rotation. The clusters of trichomes formed sheaths on the bottom of flask.

***Phormidium* sp. Kützing ex Gomont UIO 018.** Trichomes were solitary or formed clusters, straight, pale blue-green (Plate 4E), constricted at thickened cross-walls, rounded or straight at the ends. The cells were short, up to 0.7-1.4 µm wide, 1.3-3.3 µm long, the

cell contents were homogenous. Apical cells were widely rounded. Trichomes were benthic and formed sheaths in lab cultures.

***Phormidium* sp. Kützing ex Gomont UIO 145.** Trichomes were solitary or formed clusters, straight or slightly coiled (Plate 4F). Cells were long cylindrical to long with rounded ends, 0.5-1.0 µm wide, 1.5-3.7 µm long and pale blue-green. Apical cells were rounded. The trichomes were benthic and formed sheath like colonies.

***Phormidium* sp. Kützing ex Gomont UIO 146.** Trichomes were solitary or formed clusters, straight or slightly coiled, distinctly constricted at the cross-walls, not attached at the ends (Plate 4G). The cells were long cylindrical with rounded ends, 0.6-1.0 µm wide and 1.5-2.6 µm long, pale blue-green. Apical cell was rounded. The trichomes were benthic and formed sheath like colonies.

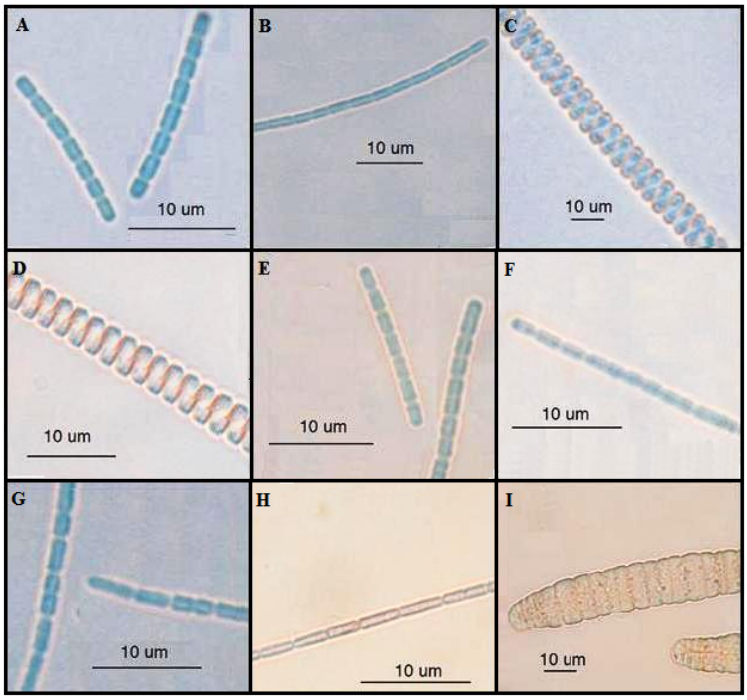


Plate 4. Light micrographs of studied strains of Oscillatoriales.
A) *Pseudoanabaena* sp. NIVA-CYA 333
B) *Pseudoanabaena* sp. NIVA-CYA 280
C) *Spirulina subsala* NIVA-CYA 163
D) *Spirulina subsalsa* NIVA-CYA 164
E) *Phormidium* sp. UIO 018
F) *Phormidium* sp. UIO 145
G) *Phormidium* sp. UIO 146
H) *Oscillatoria* sp. UIO 017
I) *Oscillatoria* cf. *chalybea* NIVA-CYA 165

***Oscillatoria* sp. Vaucher ex Gomont UIO 017.** Thallus was very thin, finely membranous, pale reddish to pinkish, spread over bottom of flask. Sheaths were thin, diffluent. Trichomes were many-celled, clearly constricted at cross-walls, motile, surrounded by a thin mucilage (Plate 4H). Cells were cylindrical, 19.2-93.8 µm wide, 78.8-159.0 µm long; cell contents were homogenous and pale reddish.

***Oscillatoria* cf. *chalybea* (Merteens ex Gomont) Anagnostidis and Komárek NIVA-CYA 165.** The filaments of *Oscillatoria* cf. *chalybea* or *Phormidium* cf. *chalybeum* were blue-green, soft and fragile (Plate 4I). Trichomes were straight or curved, slightly irregular and long. The filaments were 6.5-9.7 µm wide and 1.8-3.7 µm long. They were moving by slow oscillating and rotating movements. Cells contents were finely granulated and also with prominent large granules. Apical cells were widely rounded and without calyptra or thickened outer cell wall. The trichomes were epiphytic and benthic on the bottom of the flask.

3.4.2. Pakistani strains

Chroococcales

The identification was based on Komárek and Anagnostidis (1999).

***Synechocystis* sp. Sauvageau UK-G-102.** The cells were solitary, bright green in color, without mucilage, thallus indefinite among other micro algae. The cells were spherical, 2.4-3.1 µm x 2.2-3.3 µm, single or together (Plate 5A) and non-motile. The cell contents were blue-green and homogenous. Under laboratory conditions they formed green and thin layer on the bottom of flask.

***Chroococcus* sp. Nageli UK-G-103.** The cells were drak brown in color, cells ± spherical, 4.7-6.5 x 4.5-6.9 µm in size, after division they grouped in small colonies of 2-4 individuals, sometimes 8-16 in a gelatinous or mucous matrix (Plate 5B). During laboratory culturing they formed thick layer of colonies on the bottom of flask.

Oscillatoriales

The identification wass based on Komárek and Anagnostidis (2005) and Desikachary (1959).

***Pseudoanabaena* sp. Lauterborn UK-O-109.** Cells were blue-green in color, mucilaginous sheath absent (Plate 5C), formed small colonies, 2.3-4.1 µm broad and 2.5-4.9 µm long. Filaments were long and non-motile. Apical cell was non-capitate and rounded. Under laboratory conditions they were grown on bottom of flasks in the form of dense sheaths. On shaking they were mixed thoroughly in culturing medium.

***Pseudoanabaena* sp. Lauterborn UK-O-101.** Thallus was bright green, large filaments that were somewhat coiled and showed gliding movements. The cells were 1.8-3.0 µm broad and 2.1-4.0 µm long and constricted at the ends. The cells were non-capitate and longer than broad (Plate 5D). Sheaths were not present. Under laboratory condition they were grown on the bottom of flasks in the form of thick and compact colonies.

***Geitlerinema* sp. (Anagnostidis et Komárek) Anagnostidis UK-G-106.** Filaments were motile, bright green in color. Trichomes were thin, delicate, cylindrical, straight sometimes curved and without sheath (Plate 5E). The cells were 4.5-10.5 µm wider and 5.2-2.7 µm long. Apical cells were rounded and straight. The trichomes formed thin sheath on the bottom of flask.

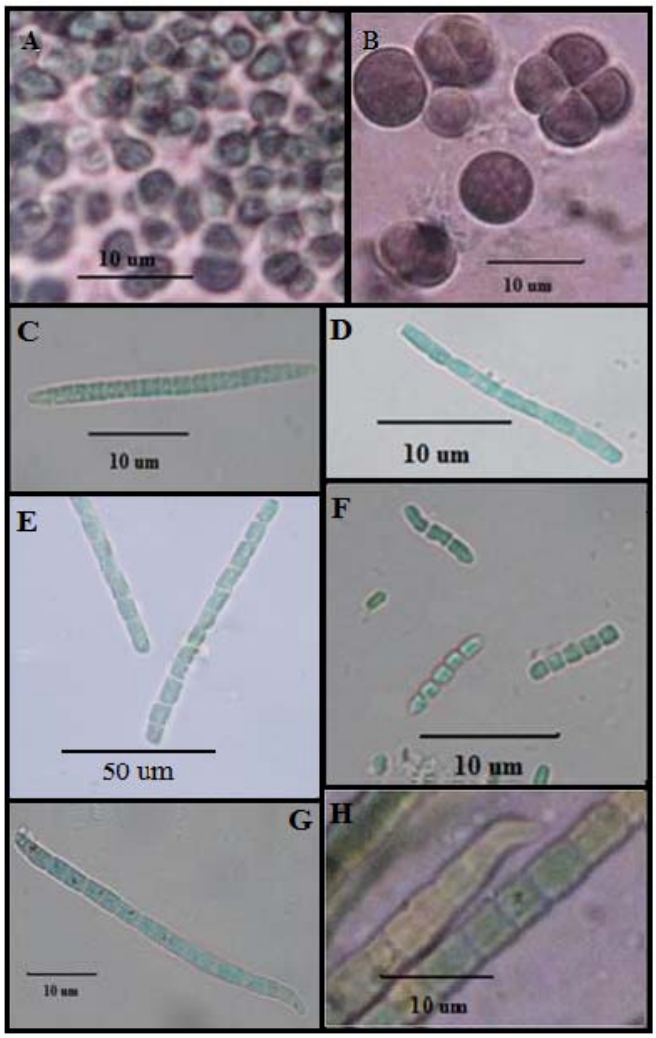


Plate 5. Light micrographs of studied Pakistani strains.
A) *Synechocystis* sp. UK-G-102
B) *Chroococcus* sp. UK-G-103
C) *Pseudoanabaena* sp. UK-O-109
D) *Pseudoanabaena* sp. UK-O-101
E) *Geitlerinema* sp. UK-G-106
F) *Chlorogleopsis* sp. UK-O-105
G) *Oscillatoria* sp. UK-G-110
H) *Oscillatoria* sp. UK-G-108

***Chlorogleopsis* sp. Desikachary UK-O-105.** The filamentous thallus was dark green. Sheath was absent (Plate 5F). The cells were 1.3-2.0 μm broad and 1.5-3.4 μm long. Apical cells were non-capital and non-motile. In laboratory cultures they grew on the bottom of culturing flasks.

***Oscillatoria* sp. Vaucher ex Gomont UK-G-110.** The filaments were green and motile. The cells were 2.9-3.6 μm broad and 4.0-7.0 μm long and filaments layed side by side. Trichomes were straight and composed of disc shaped cells. There was a little construction between trichomes. A thin sheath was present (Plate 5G). In culturing flasks they creped on the walls and formed thick layer of filaments.

***Oscillatoria* sp. Vaucher ex Gomont UK-G-108.** Thallus was green and thin membranaceous. Filaments were arranged side by side in the stratum. Trichomes were straight, elongated, erected, fragile and rapidly moving. The cells were 3.0-4.0 μm broad and 2.3-4.3 μm long. Apices of trichomes were straight, endings were sharply pointed, hooked or twisted. The cells were shorter than broad (Plate 5H). They formed thick layer of filaments under the laboratory conditions.

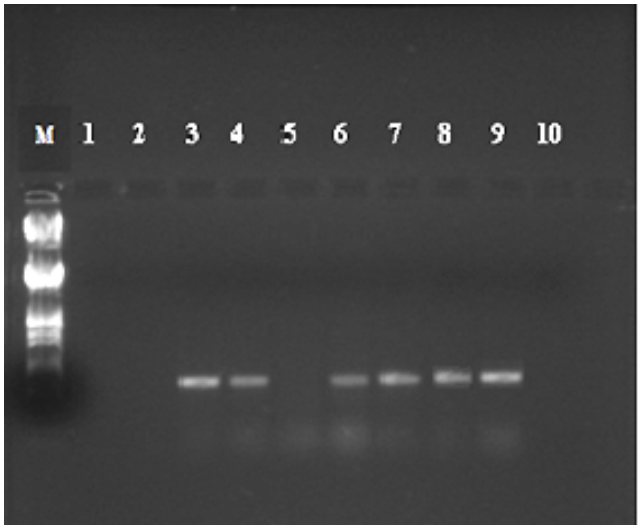
3.5. DNA sequences and molecular systematics

The systematic position of a total of 11 cyanobacterial strains were examined by investigating parts of the 16S rDNA (SSU) and phycocyanin (*cpc*BA DNA-region) genes. Initially several sets of primers and primerannealing temperatures were tested (Appendix 4).

3.5.1. Partial phycocyanin DNA-region (*cpc*BA)

To target *cpc*BA (phycocyanin DNA-region) the DNA was successfully amplified from 6 strains represented by *Synechococcus* sp., *Pseudoanabaena* spp. and *Phormidium* spp. The use of primers PCβF and PCαR at 50 °C primerannealing temperature was ideal to amplify DNA from these cells. The other set of primers (*cpc*BF and *cpc*AR) gave less amount of PCR-product under the conditions tested. The cyanobacterial strains, *Phormidium* spp. UIO 145, UIO 146, UIO 018, *Oscillatoria* sp. UIO 017, *Synechococcus* sp. UIO 013, *Pseudoanabaena* sp. NIVA-CYA 333 gave clear PCR amplification bands of phycocyanin DNA-region (Fig. 6). The PCR amplification bands of other strains are shown in Appendix 5.

Fig. 6. PCR amplification bands of partial phycocyanin DNA-region (*cpc*BA) from Norwegian cyanobacterial strain at 50 °C primer annealing temperature. The lane M is DNA size marker (Eco RI/Hind III) and the bands in 3-9 lanes correspond to the cyanobacterial strains *Phormidium* sp. UIO 145, *Phormidium* sp. UIO146, *Phormidium* sp. UIO 018, *Oscillatoria* sp. UIO 017, *Synechococcus* sp. UIO 013, *Pseudoanabaena* sp. NIVA-CYA 333 and 10 lane is PCR water as a negative control.



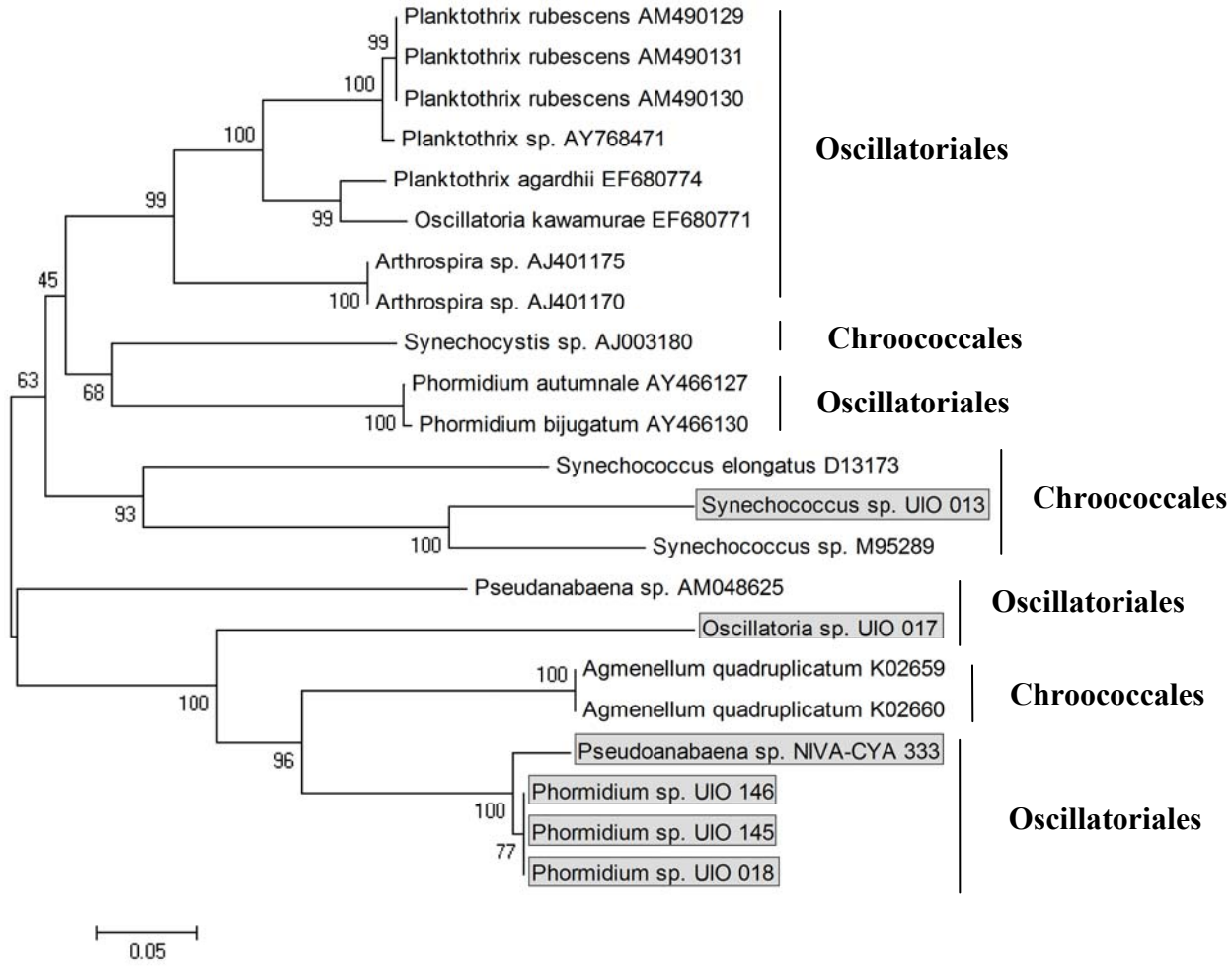


Fig. 7. Phylogenetic tree based on partial phycocyanin DNA-region (*cpc*BA) sequences, showing the relationships among some strains of Chroococcales and Oscillatoriales and their similar sequence from GenBank. The scale marker represents 0.05 nucleotide substitutions. The phylogenetic tree was inferred using the neighbor-joining method in the software Mega 4.1. Bootstrap support values are shown at the nodes of tree.

The sequences of partial phycocyanin DNA-region (*cpc*BA) were compared with those of similar cyanobacteria sequences available in GenBank and found by BLAST search (the alignment is shown in Appendix 7). Phylogenetic trees were inferred by both neighbor-joining (NJ) and minimum evolution (ME) methods. The NJ and ME methods resulted in trees that revealed good congruence with morphologically based classification for the orders Chroococcales and Oscillatoriales (Fig. 7). The strains of Chroococcales (*Agmenellum quadruplicatum* K02659 and K02660) and Oscillatoriales (*Phormidium* sp. UIO 018, UIO 145 and UIO 146 and *Pseudoanabaena* sp. CYA-NIVA 333) were clustered and supported by a 96% bootstrap value. The cyanobacterium, *Synechococcus* sp. UIO 013 clustered with GenBank *Synechococcus* sp. M95289. *Oscillatoria* sp. UIO 017 clustered with *A. quadruplicatum* (K02659 and K02660) and *Pseudoanabaena* sp. NIVA-CYA 333 with *Phormidium* spp. UIO 018, UIO 145 and UIO 146 (Fig. 7).

3.5.2. Partial 16S rDNA-region

The partial 16S rDNA-region was successfully amplified from 11 strains including representatives of *Synechococcus* spp. (NIVA-CYA 328, UIO 016, UIO 013 and UIO 012), *Geitlerinema* sp. UK-G-106, *Pseudoanabaena* spp. (NIVA-CYA 333 and NIVA-CYA 280), *Oscillatoria* sp. UIO 017 and *Phormidium* spp. (UIO 018, UIO 145 and UIO 146). For 16S rDNA 4 sets of primers were tested and used with success (Appendix 4). An experimental electrophoresis was run to note the effectiveness of different primerannealing temperatures with different sets of oligonecleotide primers. The set of primers CYA106F and CYA781R(a) at 55 °C primerannealing temperature was most successful. The sets of primers CYA106F and CYA781R(a and b) at 50, 55 and 60 °C gave a long fragment of 16S rDNA (675 bp), while another set of primers CYA359F and CYA781R(a and b) at 50, 55 and 60 °C gave a short fragment of 16S rDNA (422 bp; Fig. 8). Appendix 6 shows clear PCR amplification bands on electrophoresis gel of all strains.

The 16S rDNA sequences were compared with those of similar cyanobacterial sequences obtained by a BLAST search and available in GenBank. The alignments of 16S rDNA sequences do not show much variation within different strains within a species. *Pseudoanabaena* spp. NIVA-CYA 280 and NIVA-CYA 333 were shown identical base pairs (bp). The *Phormidium* spp. UIO 018, UIO 145 and UIO 146 were identical to a sequence of *Phormidium* sp. AB183567, downloaded from GenBank. While *Synechococcus* strains NIVA-CYA 328, UIO 012, UIO 016 and UIO 013 were clearly

different. The *Geitlerinema* sp. UK-G-106 was identical with the strains of *Geitlerinema* AY274821 and AY 274620 from GenBank (Fig. 9).

Phylogenetic trees for 16S rDNA were constructed by both neighbor-joining (NJ) and minimum evolution (ME) methods. The NJ and ME methods revealed good congruence with morphologically based classification for the orders Chroococcales and Oscillatoriales (Fig. 9). The sequences of *Oscillatoria* sp. UIO 017 did, however, not cluster with other

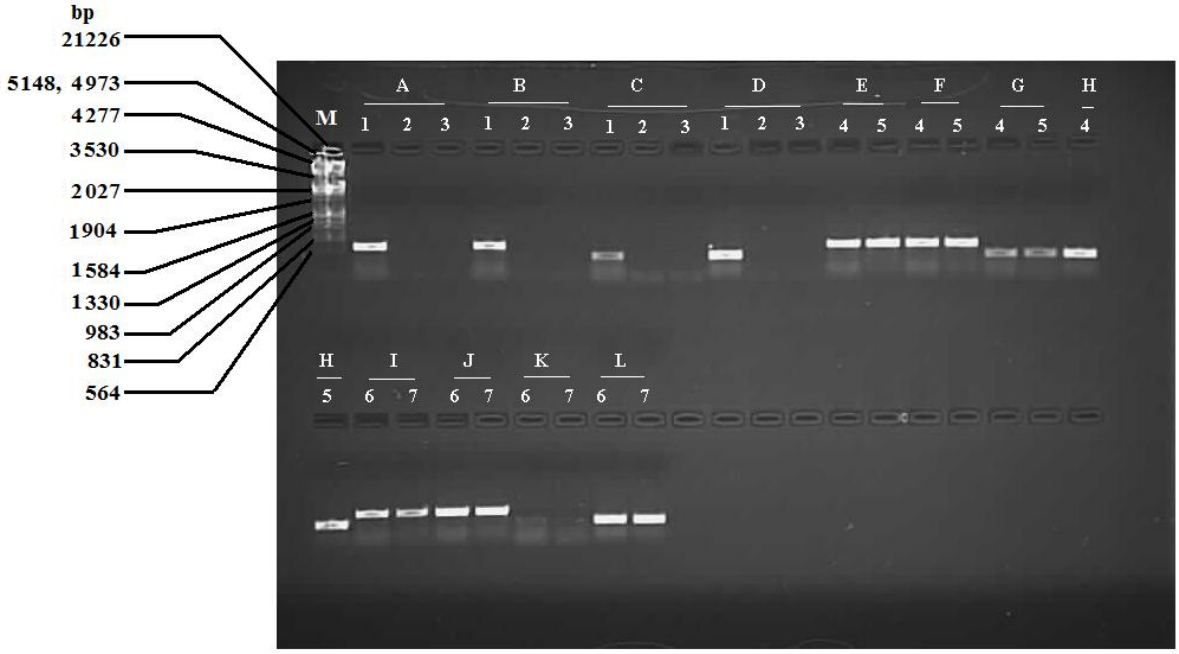


Fig.8. PCR amplification bands on electrophoresis gel of partial 16S rDNA-region at different primer annealing temperatures with different sets of primers. The lane M is DNA size marker (Eco RI/Hind III). The bands in lanes from 1-7 represent: *Synechococcus* sp. UIO 012 (lane 1, 4, 6), *Phormidium* sp. UIO 146 (lane 2, 5 and 7) and PCR water (lane 3; negative control).

Set A was at 50°C with CYA106F and CYA781R(a)
Set B was at 50 °C with CYA106F and CYA781R(b)
Set C was at 50 °C with CYA359F and CYA781R(a)
Set D was at 50 °C with CYA359F and CYA781R(b)
Set E was at 55 °C with CYA106F and CYA781R(a)
Set F was at 55 °C with CYA106F and CYA781R(b)
Set G was at 55 °C with CYA359F and CYA781R(a)
Set H was at 55 °C with CYA359F and CYA781R(b)
Set I was at 60 °C with CYA106F and CYA781R(a)
Set J was at 60 °C with CYA106F and CYA781R(b)
Set K was at 60 °C with CYA359F and CYA781R(a)
Set L was at 60 °C with CYA359F and CYA781R(b)

Oscillatoria spp. (AB003164 and AY768406) from GenBank, indicating that it belongs to a different genus. *Oscillatoria* sp. UIO 017 was different to all available sequences in DNA databases. Also, this strain is thin filamentous and red in contrast to *Oscillatoria* cf. *chalybea* NIVA-CYA 165 that was broad filamentous and blue-green in color. The phylogenetic tree of 16S rDNA gene (Fig. 9) depicts that the species of Chroococcales and Oscillatoriales were found with long distance between both orders. The tree also gave a lineage of Chroococcales which was formed by strains of *Synechococcus*. The *Synechococcus* assemblage was strongly subdivided into 3 clades. *Pseudoanabaena* spp. NIVA-CYA 280 and NIVA-CYA 333 formed a clade with sequences of *Phormidium* sp. (AB183567) and *Oscillatoria* sp.

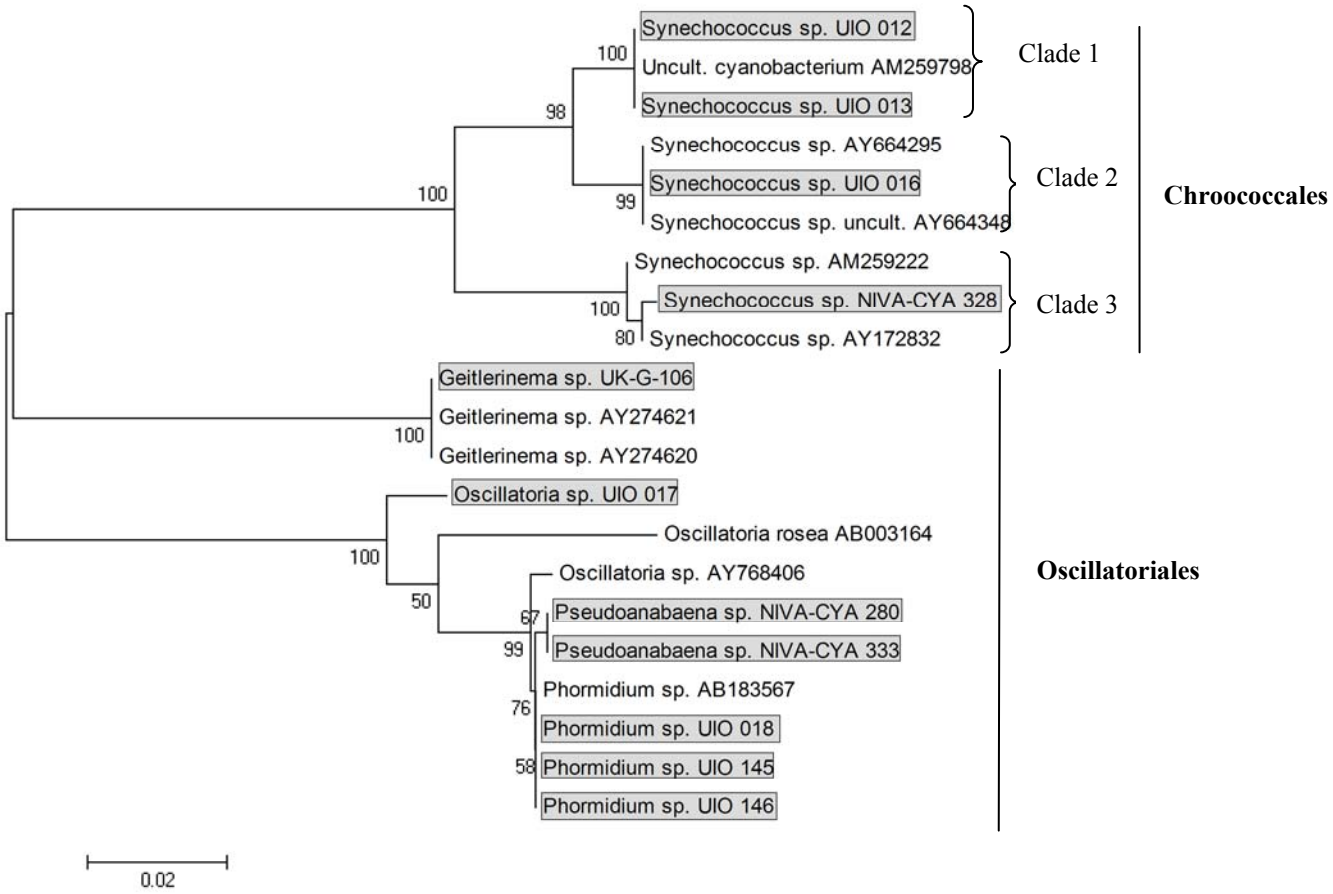


Fig. 9. Phylogenetic tree based on 16S rDNA sequences showing the relationships among studied strains of Chroococcales and Oscillatoriales and their similar sequences from GenBank. The scale marker represents 0·02 nucleotide substitutions. The phylogenetic tree was inferred using the neighbor-joining option in the software Mega 4.1. Bootstrap support values are shown at the nodes of tree.

Table 8. Length of base pairs (bp) of DNA sequences and accession numbers of studied .
(-) indicates strains that were not sequenced successfully.
(xxxxxx) applied for accession nos.

Taxa	Strain codes	Length in base pairs (bp) in partial phycocyanin DNA-region (*cpc* BA)	Accession no.	Length in base pairs (bp) of partil 16S rDNA	Accession no.
Chroococcales					
Synechococcus sp.	NIVA-CYA 328	-	-	577	FM877959
Synechococcus sp.	UIO 012	-	-	624	FM877960
Synechococcus sp.	UIO 016	-	-	623	FM877961
Synechococcus sp.	UIO 013	587	xxxxxx	644	FM877962
Oscillatoriales	**Oscillatoriales**				
Pseudoanabaena sp.	NIVA-CYA 333	625	xxxxxx	608	FM877958
Pseudoanabaena sp.	NIVA-CYA 280	-		576	FM877957
Phormidium sp.	UIO 018	602	xxxxxx	574	FM877953
Oscillatoria sp.	UIO 017	637	xxxxxx	611	FM877954
Phormidium sp.	UIO 145	615	xxxxxx	605	FM877955
Phormidium sp.	UIO 146	613	xxxxxx	624	FM877956
Geitlerinema sp.	UK-G-106	-	-	566	FM877963

(AY68406) with 99% of bootstrap support. The alignment of the DNA sequences partial 16S rDNA-region of all studied cyanobacteria and their similar strains from GenBank are shown in Appendix 8. *Gietlerinema* sp. UK-G-106 was represented 100% similarity with other starins of *Geitlerinema* spp. AY274621 and AY274620 from Genbank.

The length in base pairs (bp) of DNA sequences and GenBank accession numbers for sequences determined in this study are shown in Table 8. The length of sequences is without gaps.

3.5.3. Phylogeny of *Geitlerinema* sp. UK-G-106 on the basis of partial 16S rDNA-region

Phylogenetic trees for 16S rDNA were constructed by both neighbor-joining (NJ) and minimum evolution (ME) methods. The NJ and ME methods revealed good congruence with morphologically based classification for *Geitlerinema* sp. UK-G-106 (Fig. 10). The sequences of *Geitlerinema* sp. UK-G-106 was identical to the sequences of their 8 similar strains of *Geitlerinema* from GenBank (Fig. 10). It shows the geographical distribution of *Geitlerinema* spp. The *Geitlerinema* sp. UK-G-106 was also matched with its similar sequences of uncultured *Geitlerinema* spp. AY874006 and AY874007 and *Geitlerinema* sp.

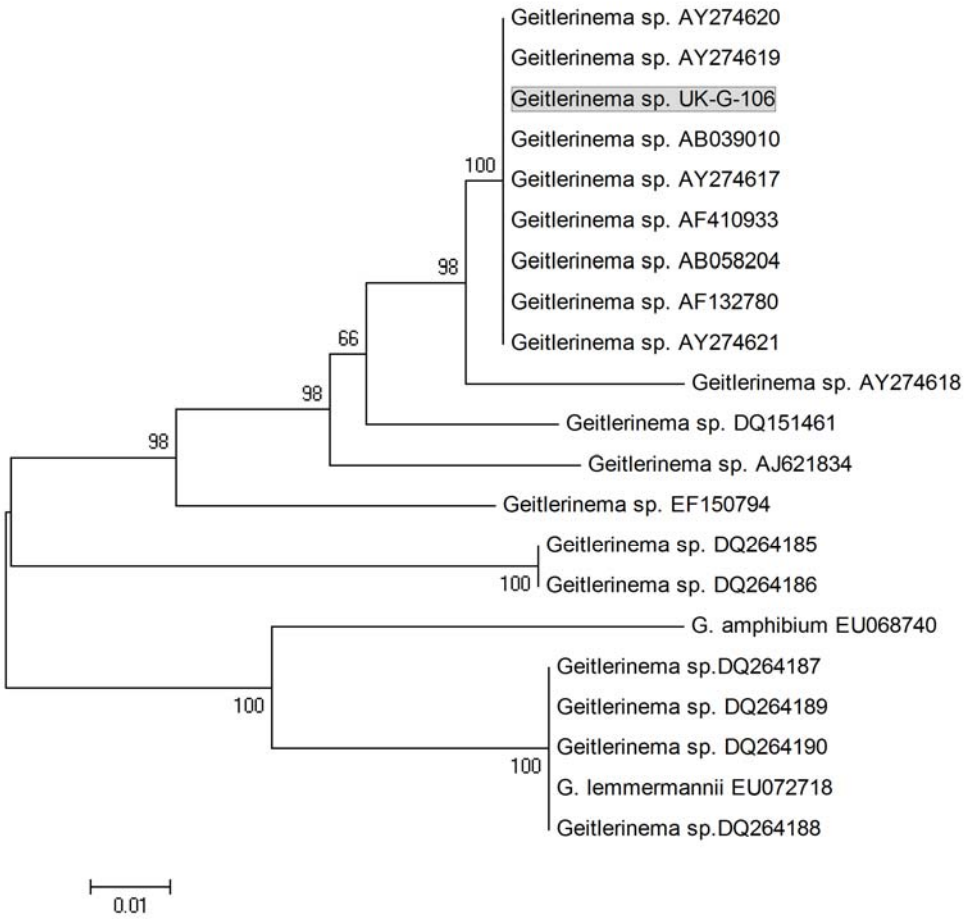


Fig. 10. Phylogenetic tree based on 16S rDNA sequences showing the relationships among studied strain *Geitlerinema* sp. UK-G-106 and its similar sequences from GenBank. The scale marker represents 2.0 nucleotide substitutions. The phylogenetic tree was inferred using the neighbor-joining option in the software Mega 4.1. Bootstrap support values are shown at the nodes of tree.

AF317510, AF473908 and AY426548, from GenBank but these sequences were too short and not possible to align reliably that's why they were deleted from the tree. The alignment of the DNA sequences (partial 16S rDNA-region) of studied *Geitlerinema* sp. UK-G-106 and its similar strains from GenBank are represented in Appendix 9.

3.6. *Artemia* assay

A total of 24 strains (Table 7) were tested for toxicity to *Artemia franciscana* nauplii.

The crude extracts of all strains except one did not show any toxicity to the *A. franciscana* nauplii. Only crude extract of *Geitlerinema* sp. UK-G-106 showed lethality to *A. franciscana*. This assay revealed that the degree of lethality against cyanobacterial crude

extract of *Geitlerinema* sp. UK-G-106 was related to dose, and showed remarkable toxicity even at very low concentration (Appendix 11). Figures 11A and 11B show probit transformed mortality of *A. franciscana* as a function of concentration of crude extract of *Geitlerinema* sp. UK-G-106 at different concentrations and its LC_{50} values in Table 9. Figure 11C shows combination of Figs. 11A and 11B. The fractionation with methanol:water (MeOH:H_2O; v/v) of crude extract of *Geitlerinema* sp. UK-G-106 revealed that the percentage of mortality decreases as concentration of methanol (MeOH) increases (Appendix 12). Figures 12A-F present a relationship between probit transformed mortality of *A. franciscana* as a function of concentration of *Geitlerinema* sp. UK-G-106 at different concentrations of MeOH. As the percentage of MeOH increases the value of LC_{50} also increases (7.22 mg dw mL^{-1}, Table 9) showing that the toxicity decreases.

Figures 13A-C and 14 depict a relation between probit mortality of *A. franciscana* and concentration of freshwater strains as positive controls *P. rubescens* NIVA-CYA 407, *Planktothrix agardhii* NIVA-CYA 229 and *Microcystis aeruginosa* NIVA-CYA 166 and sodium dodecly sulfate (SDS; standard toxin), respectively.

The percentage of mortality of *Artemia* nauplii by the positive controls of freshwater strains i.e. *P. rubescens* NIVA-CYA 407*, P. agardhii* NIVA-CYA 229 and *M. aeruginosa* NIVA-CYA 166 showed 0.05-0.12 mg dw mL^{-1} LC_{50} values (Table 10; Appendix 10). The positive standard sodium dodecyl sulfate (SDS; Appendices 13 and 14) showed a LC_{50} value of 17.85 mg L^{-1} (Table 10). The results showed that using concentrations of 1, 0.1, 0.01, 0.001 mg dw mL^{-1} of a crude extract of *Geitlerinema* sp. UK-G-106 showed 0.016 mg dw mL^{-1} LC_{50} (Table 9).

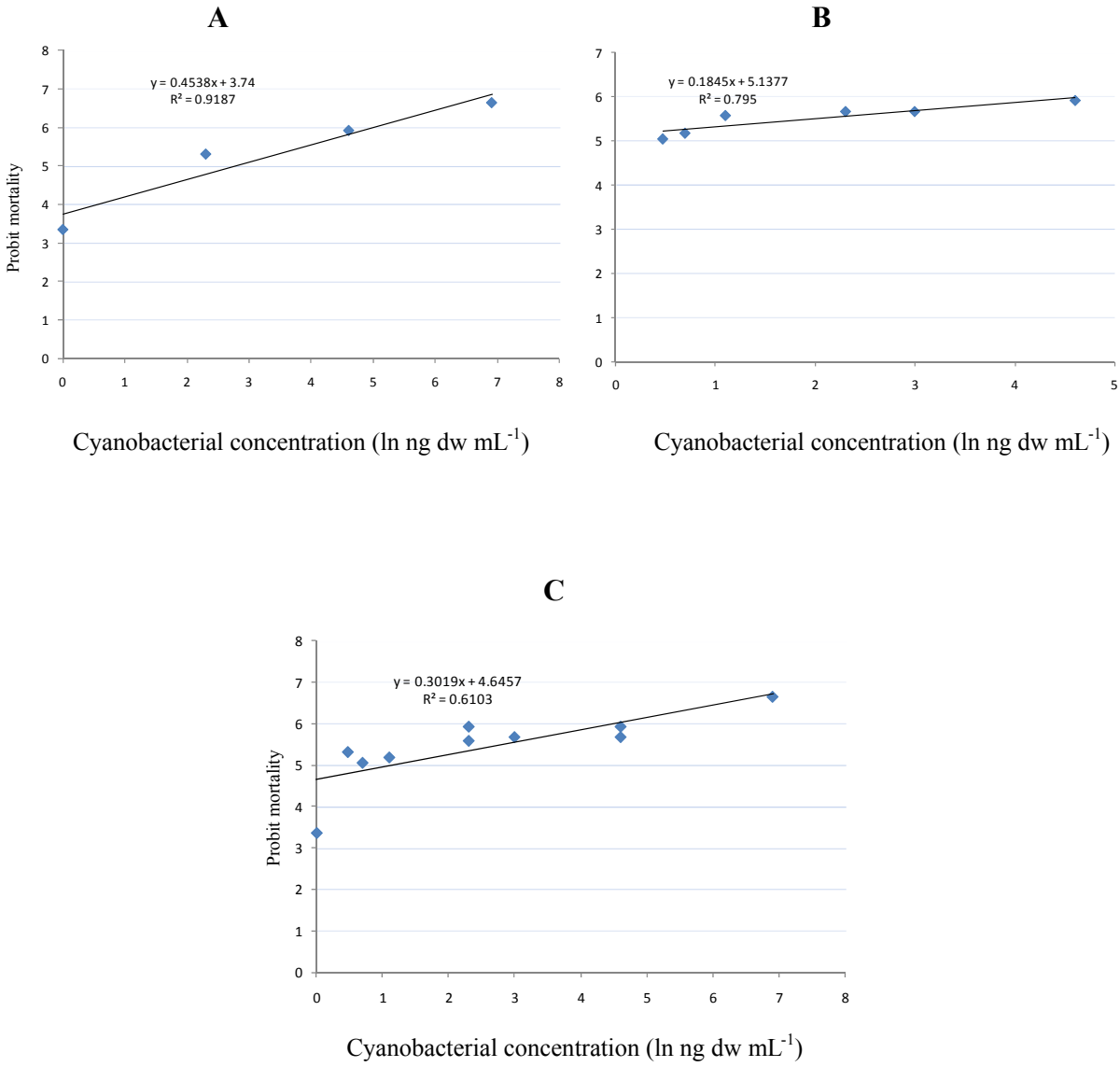


Fig. 11. The probit transformed mortality of *Artemia franciscana* as a function of concentration of a total crude extract of the cyanobacterium *Geitlerinema* sp. UK-G-106 in three separate experiments.
A) With concentrations of 1, 0.1, 0.01, 0.001 mg dw mL^{-1}
B) With concentrations of 0.1, 0.02, 0.01, 0.003, 0.002, 0.0016 mg dw mL^{-1}
C) Combination of concentrations of Figs. 5A and 5B.

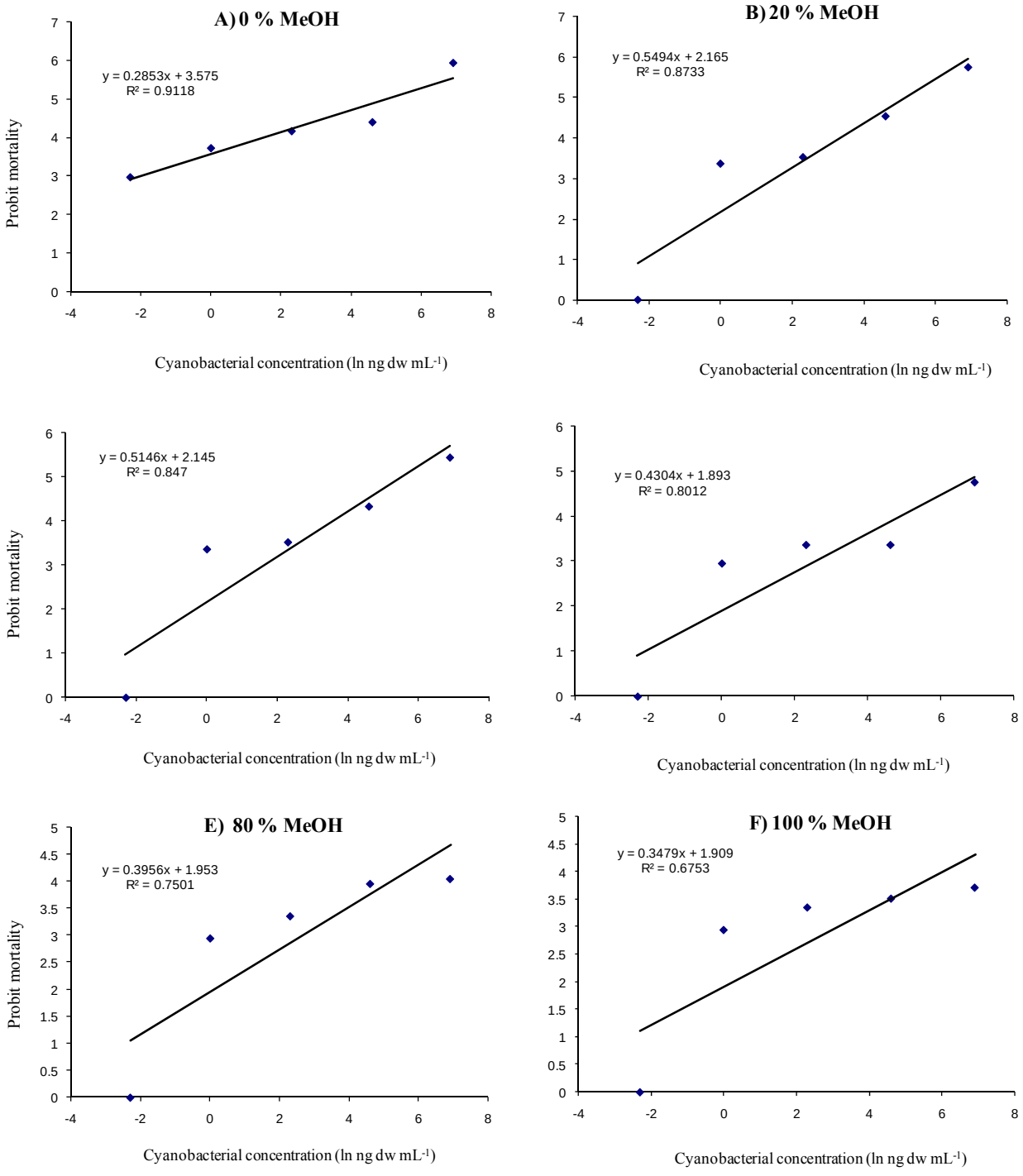
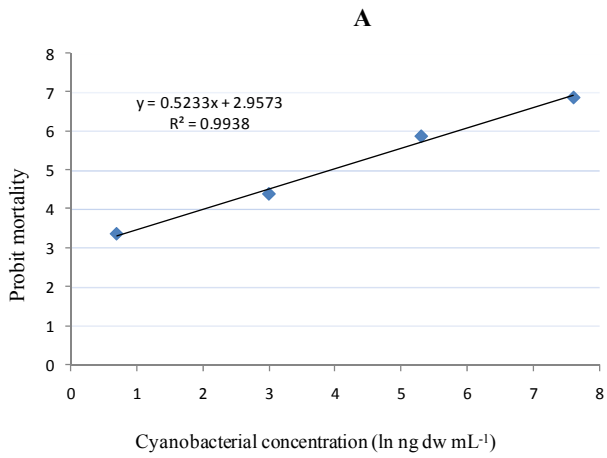
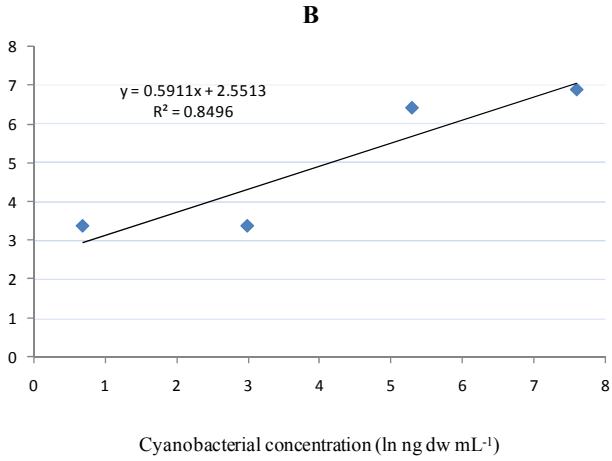


Fig. 12. The probit transformed mortality of *Artemia franciscana* as a function of concentration of the cyanobacterium *Geitlerinema* sp. UK-G-106 at different ratios of MeOH:H_2O.





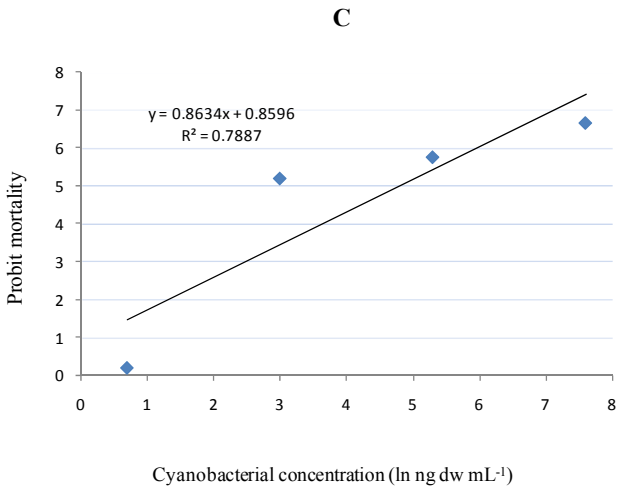


Fig. 13. The probit transformed mortality of *A. franciscana* as a function of concentration of the freshwater cyanobacteria of A) *P. rubescens* NIVA-CYA 407, B) *P. agardhii* NIVA-CYA 229 and C) *M. aeruginosa* NIVA-CYA 166 (positive controls).

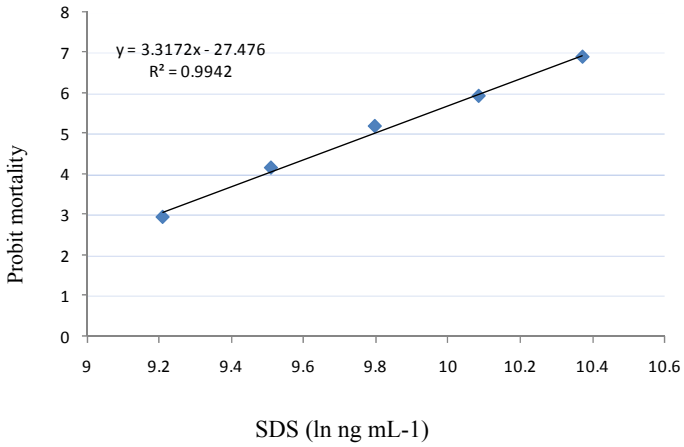


Fig. 14. The probit transformed mortality of *A. franciscana* as a function of concentration of sodium dodecyl sulfate (SDS; standard toxin) against mortality of *A. franciscana* nauplii.

Table 9. Toxicity (LC_{50} values) to *Artemia* exposed with crude extract (1 mg dw mL^{-1}) in stock and with MeOH:H2O fractions of *Geitlerinema* sp. UK-G-106.

Exposure to	LC_{50} - 24 h (mg dw mL^{-1})
Total crude extract	
1) at 1, 0.1, 0.01, 0.001	0.016
2) at 0.1, 0.02, 0.01, 0.003, 0.002, 0.0016	0.0005
Combination of 1 and 2	0.0032
Fractions	
0% MeOH	0.15
20% MeOH	0.17
40% MeOH	0.25
60% MeOH	1.3
80% MeOH	2.21
100% MeOH	7.22

Table 10. Concentrations and LC_{50}-24 h (mg dw mL^{-1}) values of freshwater strains and SDS as positive controls.

Controls	Strain codes	LC_{50} - 24 h (mg dw mL^{-1})
[1]*Planktothrix rubescens*	NIVA-CYA 407	0.05
[1]*Planktothrix agardhii*	NIVA-CYA 229	0.06
[2]*Microcystis aeruginosa*	NIVA-CYA 166	0.12
Standard toxin		**(mg mL^{-1})**
[3]SDS (sodium dodecyl sulfate)		17.85

[1]at the concentration of 1, 0.1, 0.01, 0.001 mg dw mL^{-1}

[2] at the concentration of 2, 0.2, 0.02, 0.002 mg dw mL^{-1}

[3] at the concentration of 1, 1.35, 1.8, 2.4, 3.2 mg dw mL^{-1}

Extract from *Geitlerinema* sp. UK-G-106 was also tested for ability to induce cell death at the University of Bergen. According to Linn Oftedal (personal communication) the extract induced programmed cell death (apoptosis) in rat promyelogenous leukaemia cells (IPC-81 wt) and the observed cell death was not caused by adenosine.

3.7. Oligopeptide analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS)

These results were obtained by Thomas Rohrlack at NIVA. The procedure was applied to analyze the 24 cyanobacterial samples (16 species from Norwegian and 8 species from Pakistani waters) to detect the presence of oligopeptides in molecular mass ranging from 500-2000 Da. Only 3 species, *Geitlerinema* sp. UK-G-106, *Synechococcus* sp. UIO 015 and *Pseudoanabaena* sp. UK-O-101 showed clear peaks in total scanning mode for this mass range (500 to 2000 Da; Figs. 15-17). Figure 15A shows a total scan of *Geitlerinema* sp. UK-G-106. In this chromatogram 4 large signals in a range 8.00-13.00 min retention time, were recorded. The Fig. 15B represents a chromatogram of *Geitlerinema* sp. UK-G-106 with 4 large and clear signals (retention time 8.47, 10.49, 11.49 and 12.69 min) in a range of 711-722 Da between 8.00-13.00 min. These peaks represent unknown oligopeptides and may show the presence of a new class of peptides. The analyses were not processed further due too low amount of cell material and that the strain was dead.

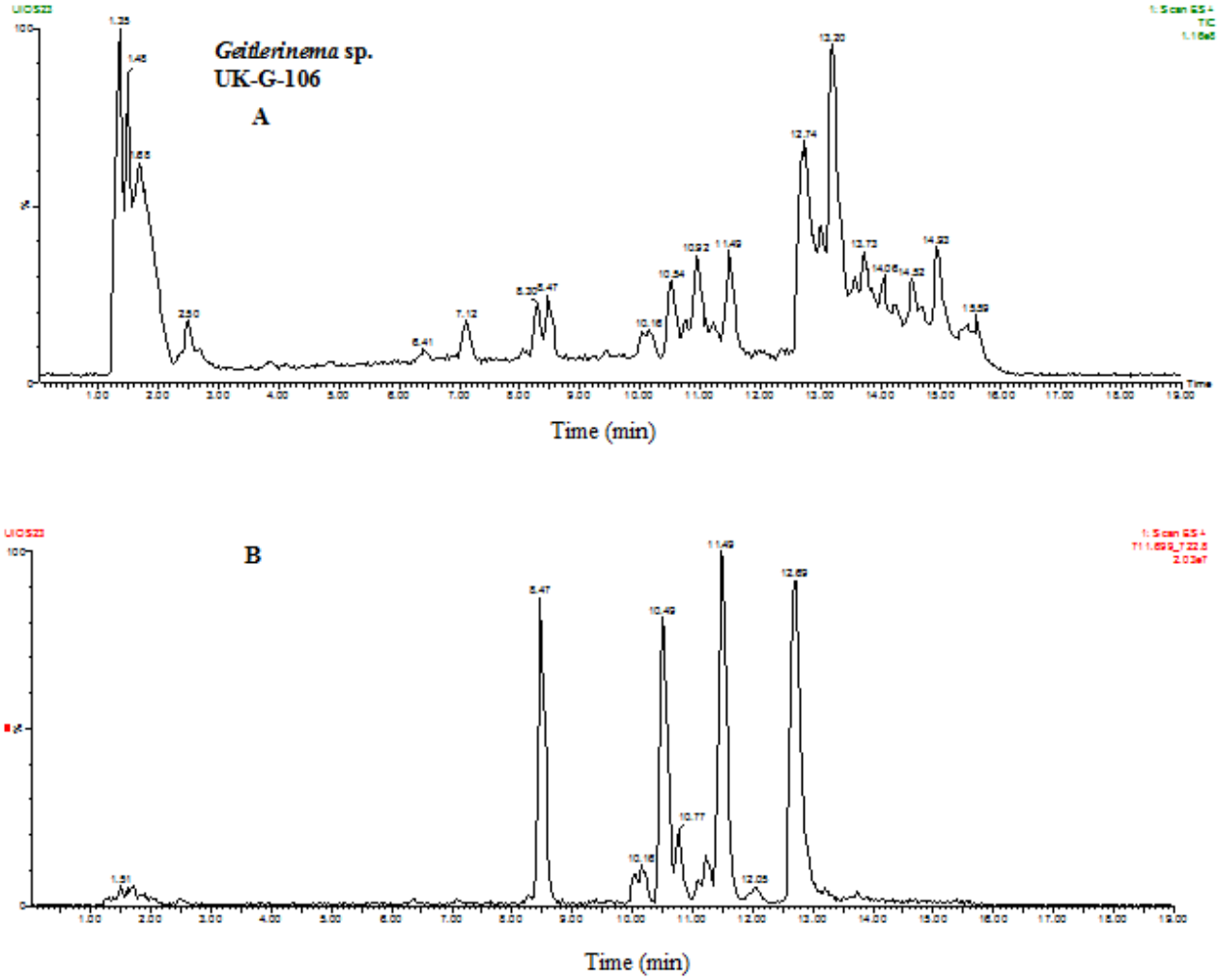
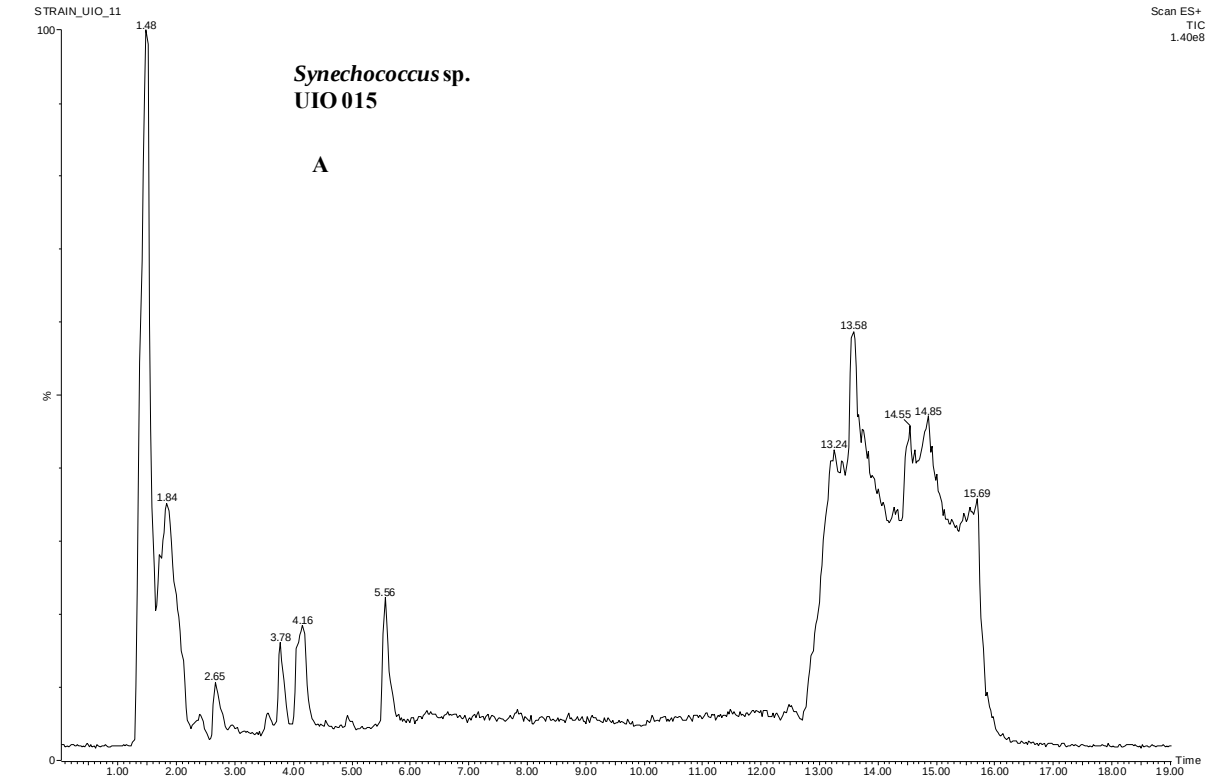
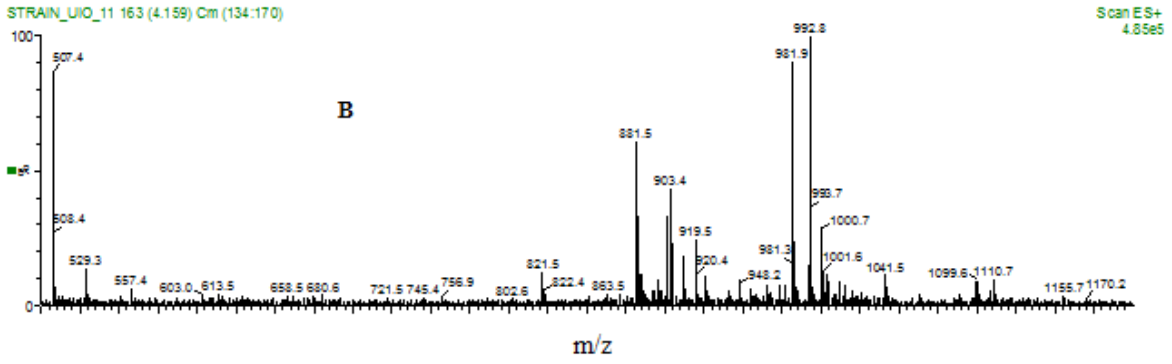


Fig. 15. Chromatograms of oligopeptide separations from *Geitlerinema* sp. UK-G-106. A- full scan and B- four clear and large peaks in the range of 8-13 min retention time.



Time (min)



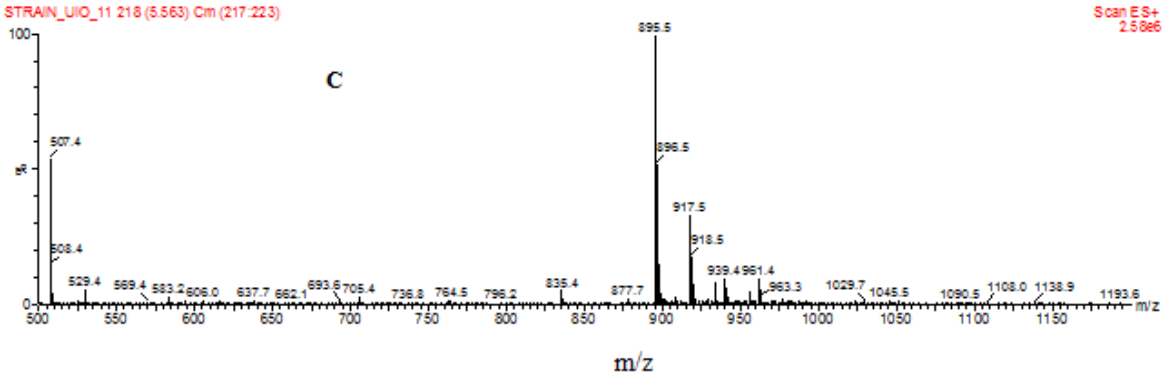


Fig. 16. Chromatogram of oligopeptide separation from *Synechococcus* sp. UIO-015. A- full scan, B and C- mass spectra.

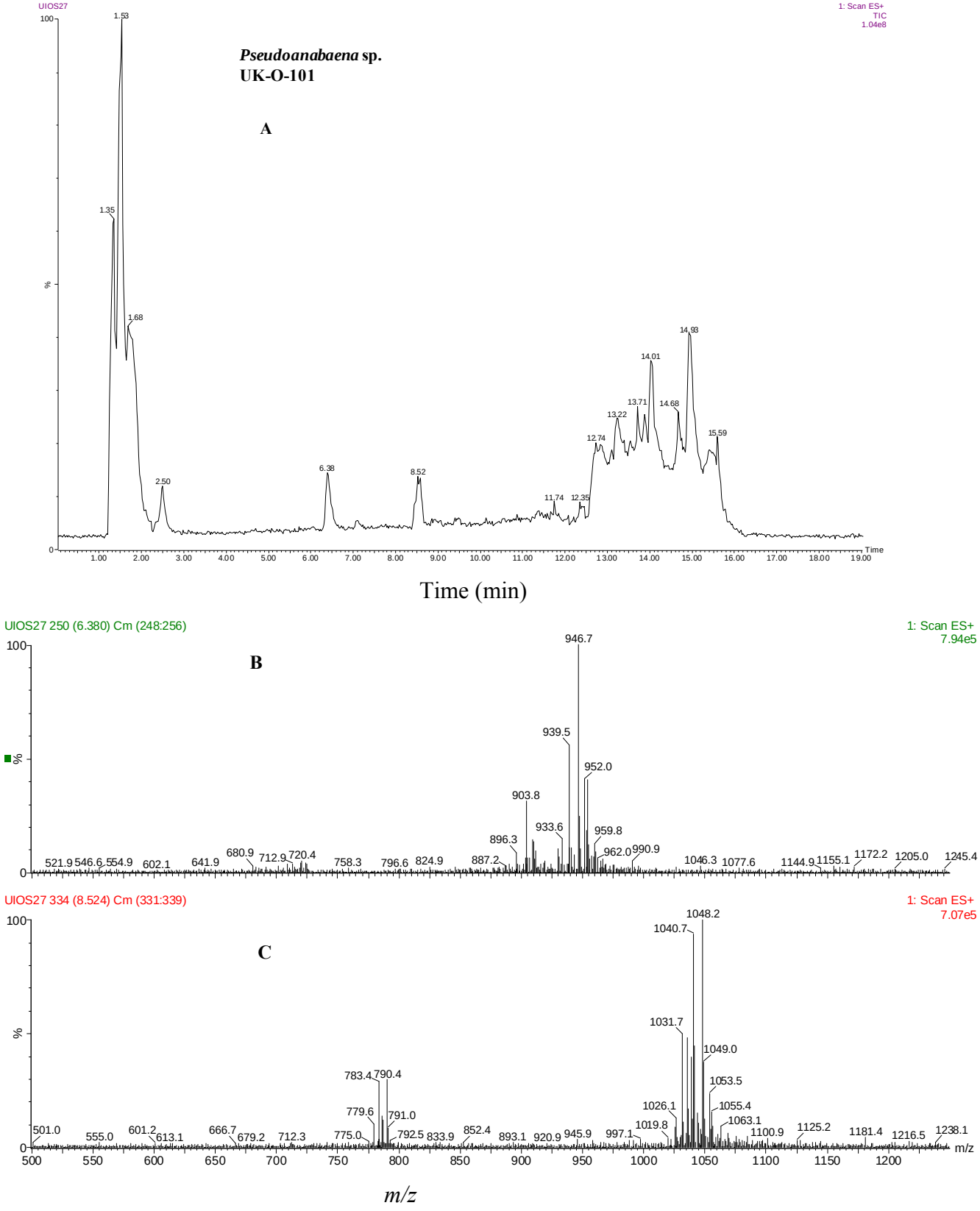


Fig. 17. Chromatograms of oligopeptide separations of *Pseudoanabaena* sp. UK-O-101. A- full scan, B- mass spectrum of cluster I and C- mass spectrum of cluster II with 2 groups of substances.

In full scan mode the cyanobacterium, *Synechococcus* sp. UIO 015 showed 4 large peaks with retention time 2.65, 3.78, 4.16 and 5.56 min. in a range of 2.5-6.00 min (Fig. 16A). The mass spectrum of *Synechococcus* sp. UIO 015 shows 2 clusters of substances (Fig. 16B) and one of the clusters represent 4 large peaks (895.5-918.5 Da) between 900-925 *m/z* (Fig. 16C).

Pseudoanabaena sp. UK-O-101 also showed clear peaks in total scanning mode from the mass range of 500 to 2000 Da. The total scan showed 2 clear clusters I and II of substances at retention time 6.38 and 8.52 min between 2.5-13.00 min (Fig. 17A). The mass spectrum (Fig. 17B) represents double charged cluster I and a group of substances with similar polarity, ranged from 900-990 *m/z*. Figure 17C shows mass spectrum (*m/z*) of clusters I and II. It represents 2 clusters ranged from 750-800 *m/z* and 1025-1075 *m/z,* respectively.

The chromatograms of other cyanobacterial strains are shown in Appendix 15.

4. DISCUSSION

In this study different methods have been used to obtain uni-algal cultures of benthic and planktonic cyanobacteria. In the present investigation it was noted that the streaking (for benthic and coccoid), capillary methods (for benthic filamentous) and the serial dilution culture (for planktonic) methods were good to isolate cyanobacteria, as previously described by Andersen and Kawachi (2005). By streaking method I could isolate strains of benthic filamentous (*Phormidium* spp. UIO 145, UIO 146, *Geitlerinema* sp., UK-G-106, *Chlorogleopsis* sp. UK-O-105, *Oscillatoria* spp. UK-G-108 and UK-G-110, *Pseudoanabaena* spp. UK-O-109 and UK-O-101). By dilution method coccoids (*Synechococcus* sp. UK-G-102 and *Chroococcus* sp., UK-G-103) were isolated. The serial dilution technique was also suitable for isolation of *Phormidium* sp. UIO 018 and *Oscillatoria* sp. UIO 017 collected from natural environment (Andersen and Kawachi 2005).The capillary method was found to be effective to obtain strains of certain filamentous *Phormidium* spp. UIO 018, UIO 145 and UIO 146 and *Oscillatoria* sp. UIO 017.

In this study several growth media were tested to obtain good growth. It was noted that the IMR ½ (30 PSU; Eppley *et al* 1967 modified by Paasche 1971) and ES (30 PSU; Provasoli 1968) were good for the growth of marine strains from Norway. Another growth medium, Z8 (16 PSU; Staub 1961 modified by Kotai 1972) was also good for brackish water strains of Norway. For Pakistani strains ASN III (34 PSU) was good (Dawood 1998). These media provided favourable conditions for studied strains and the population of strains become dense due to presence of Na^+, Mg^+ and Ca^+ ions (Rippka *et al* 1979), because without addition of nutrients and trace metals, the yield of algae become too low for laboratory experiments (Harrison and Berges 2005). During present studies it was observed that only 2 strains of *Synechococcus* NIVA-CYA 328 and UIO 012 were able to grow in all tested media (IMR ½, ES and Z8) media with different salinities (16 and 30 PSU). It depicts that *Synechococcus* spp. have the ability to grow and survive in a wide range of salinities (Blumwald *et al* 1983, Blumwald and Elisha 1984). Several studies have also been done on the production of microcystins and salinity showing that the salinity levels may be a significant factor in selecting for the microcystin produces (Carmichael and Li 2006).

In another study Burja *et al* (2002) cultured *Lyngbya majuscula* in 3 different media having different salinity, pH and trace elements. They suggested that varying the culture conditions under which *L. majuscula* was grown had the greatest effect on secondary metabolite production. Orr *et al* (2004) did an experiment on laboratory culture of *Microcystis*

aeruginosa and found that *M. aeruginosa* is more tolerant at high salinity to produce microcystin. It is suggested that the biological activity of cyanobacteria appears to be depended upon growth conditions and therefore activity of the less amount of toxin producing organisms may be improved by altering the cultural conditions. Recently, Rohrlack and Utkilen (2007) have worked on a freshwater cyanobacterium, *Planktothrix agardhii* collected from Lake Langsjon, Finland and run 3 experiments to assess the effect of light, nitrogen and phosphorus. They concluded that the cell-bound anabaenopeptins and microviridin I were associated with a high availability of nitrogen and phosphorus as similar to those previously reported for microcystins (Orr and Jones 1998).

Microscopic based identification of strains requires time and skills and sometimes it is very difficult to differentiate between certain strains of the same genus, toxic and non-toxic strains (Scholin *et al* 2003), morphological characteristics due to different culturing conditions (Castenholz and Waterbury 1989) and morphologically identical strains (Wilmotte and Herdman 2001). Lehtimäki *et al* (2000), Gugger *et al* (2002) and Rippka *et al* (2001) also mentioned that the phenotype of heterocytous cyanobacteria change their morphology during laboratory cultivation which makes the identification of strains difficult. Traditionally microscopic techniques are convenient to identify cyanobacterial strains but not for all strains especially in the case of *Pseudoanabaena* and *Phormidium* strains. In the present study during microscopic characteristics, based on morphology, it was difficult to distinguish between genera of *Pseudoanabaena* and *Phormidium,* and *Oscillatoria* and *Geitlerinema.* I misidentified *Phormidium* spp. UIO 018, UIO 145 and UIO 146 as *Pseudoanabaena* spp.; *Oscillatoria* sp. UIO 017 as *Phormidium* sp. and *Geitlerinema* sp. UK-G-106 as *Oscillatoria* sp. when I followed John *et al* (2002) and Komárek and Anagnostidis (2005).

During studies it seemed that all morphological characteristics of *Pseudoanabaena* and *Phormidium* strains were more or less the same but they were only distinguished on the basis of their motility (Komárek and Anagnostidis 2005) and aggregation of trichomes (John *et al* 2002). Komárek and Anagnostidis (2005) mentioned that *Phormidium* spp. show clear motile movements; gliding, creeping, waving, trembling while *Pseudoanabaena* spp. don't show clear motility. They also depicted that the taxonomy of *Phormidium* belongs to the most difficult cyanoprokaryotic genera as it comprises of numerous morphotypes with many transient forms. According to John *et al* (2002) many trichomes of *Phormidium* spp. form gelatinous or leathery mats while filaments of *Pseudoanabaena* spp. don't gather (in this study the identification of *Phormidium* spp. was also confirmed by DNA analyses). During this study I found 3 strains of *Phormidium* from different collection sites (UIO 018 from

Huk, Oslofjord in 2007; UIO 145 from Oslofjord, Hulvika in 2006 and UIO 146 from Northland Kaupang in 2006) and no strain of *Pseudoanabaena* was found. It has also been mentioned in previous literature from Norwegian waters that *Phormidium* spp. are more common as compared to *Pseudoanabaena* spp. (personal communication with Prof. Jan Rueness, UiO). According to the NIVA Culture Collection of Algae a total of 30 strains of *Phormidium* and 7 strains of *Pseudoanabaena* have isolated from marine and frewshwaters of Norway.Only 8 species of marine *Phormidium* was reported from Swedish waters (Lindstedt 1943) and 8 species from Oslofjord (Wiik 1981). Both of them did not find any species of *Pseudoanabaena* from marine Norwegian waters. In Pakistani marine waters only 4 species of *Pseudoanabaena* and 30 species of *Phormidium* have been reported (RETA and IUCN report 2000). These observations show that *Phormidium* spp. are more common in aquatic environments as compared to *Pseudoanabaena* spp.

The DNA analysis is one of the most reliable methods to identify the strains by using oligonucleotide primers/probes and it also helps to identify bloom forming cyanobacteria (Neilan *et al* 1995). In this study the partial phycocyanin DNA-region (*cpc*BA) and partial 16S rDNA-region were targeted to confirm the identification of strains. This method was helpful to identify the certain strains of the present study i.e. *Phormidium* spp. UIO 018, UIO 145 and UIO 146, *Oscillatoria* sp. UIO 017 and *Geitlerinema* sp. UK-G-106. The partial phycocyanin DNA-region (*cpc*BA) was successfully targeted by the primers *cpc*BF and *cpc*AR and PCβF and PCαR (Sequences 5' to 3') as they were also useful for successful amplification of certain species of freshwater toxic cyanobacteria, *Anabaena* spp., *Aphanizomenon* spp., *Cylindrosperopsis* spp., *Microcystis* spp., *Nodularia* sp., *Nostoc* spp., *Oscillatoria* spp., *Synechococcus* sp. and *Pseudoanabaena* sp. (Neilan *et al* 1995). Some strains of freshwater *Synechococcus* spp. from USA, Japan, Finland and Ireland have also been successfully amplified by the use of sequencing primers *cpc*BF and *cpc*AR (Robertson *et al* 2001).

The sequences of partial phycocyanin DNA-region (*cpc*BA) of *Synechococcus* sp. UIO 013 was compared with cyanobacterial sequence of *Synechococcus* sp. M95289 (de Lorimier *et al* 1993) available in GenBank and found 100% similarity. The strain of Oscillaroriales (*Oscillatoria* sp. UIO 017) was found to be intermixed with *Agmenellum quadruplicatum* K02659 and K02660, a finding consistent with previous studies based on sequences of the partial phycocyanin DNA-region (*cpc*BA; Pilot and Fox 1984 and de Lorimier *et al* 1984). The cyanobacteria *Agmenellum quadruplicatum* K02659 and K02660 (Pilot and Fox 1984,

de Lorimier *et al* 1984) also showed phylogenetic relation with *Pseudoanabaena* sp. NIVA-CYA 333 and *Phormidium* spp. (UIO 018, UIO 145 and UIO 146).

During the course of study the 2 stes of primers CYA359F and CYA781R(b) and CYA106F and CYA781R(a), (sequences 5' to 3') at 50, 55 and 60°C primerannealing temperature were successful to target partial 16S rDNA-region. Nübel *et al* (1997) also used the primers CYA359F and CYA781R successfully to match 16S rDNA-region sequences in some cyanobacteria including some strains of *Cyanothece, Synechococcus, Dactylococcopsis, Aphanothece, Gleocapsa, Lyngbya, Microcoelus, Geitlerinema, Oscillatoria, Spirulina, Scytonema, Calothrix* and *Aphanozimenon.*

In this study the representatives of Chroococcales (*Synechococcus* sp. UIO 012) and Oscillatoriales (*Phormidium* sp. UIO 146) showed optimal PCR conditions at 55 °C primerannealing temperature with CYA106F and CYA781R(a). Another study shows that for some strains of *Anabaena* spp., *Aphanizomenon* spp., *Cylindrosperopsis* spp., *Microcystis* spp., *Nodularia* sp., *Nostoc* spp., *Oscillatoria* spp., *Synechococcus* sp. and *Pseudoanabaena* sp. the optimal PCR conditions were found at a primer-template annealing temperature of 55°C (Neilan *et al* 1995).

In the present investigation the cyanobacterium *Geitlerinema* sp. UK-G-106 was identical with other strains of *Geitlerinema* AY274620 and AY274621 (Ochoa *et al* 1997 and Perez-Linares 2003) from GenBank by using CYA106 F and CYA781R (a) to target partial 16S rDNA gene. The partial 16S rDNA of *Geitlerinema* sp. was also successfully targeted by the primers CYA359F and CYA781R, which match virtually all partial 16S rDNA sequences of *Geitlerinema* sp. UK-G-106 (Nübel *et al* 1997).

In this study on the basis of morphology and 16S rDNA sequence homology Norwegian *Synechococcus* spp. assemblages have been subdivided into 3 clades with their identical strains. In the first clade *Synechococcus* spp. UK-G-012 and UIO 013 showed 100% similarity with uncultured cyanobacterium AM259798 (Thiel *et al* 2007) collected from the Mediterranean Sea. In the second clade the 16S rDNA sequence of *Synechococcus* sp. UIO 016 was presented 100% similarity with *Synechococcus* spp. AY664295 and uncultured *Synechococcus* sp. AY664348 (Jiao *et al* unpublished data) from the Bering Sea. In the third clade *Synechococcus* sp. NIVA-CYA 328 showed close relation with *Synechococcus* sp. AY259222 (Rajaniemi-Wacklin *et al* 2008) from Finland and *Synechococcus* sp. AY172832 (Fuller *et al* 2003) from the Red Sea. The present study also represents that picocyanobacteria, *Synechococcus* spp. UK-G-012, UIO 013, UIO 016 and NIVA-CYA 328 are widely distributed and commonly occur in marine waters and contribute significant role

in the primary production in marine aquatic ecosystems (Stockner *et al* 1986, Partensky *et al* 1999, Vincent 2000, Becker *et al* 2004).

It is necessary to identify these strains up to species level. It appears that the classical morphological definitions of the corresponding taxa may in some cases be adequate but not upto species. Turner (1997) suggested that, although cyanobacterial 16S rDNA sequence comparisons are not suitable for resolution below the level of genus, generic assignments appear to correlate with sequence data.

Variation for *Geitlerinema* spp. within the 16S rDNA gene reflected a distinct geographic grouping of isolates. The *Geitlerinema* sp. UK-G-106 nucleotide sequences from Pakistan was identical and 100 % similar with other *Geitlerinema* spp. AY274621 reported from Pacific coastline of the California Peninsula during 1992-1995 (Ochoa *et al* 1997) and *Geitlerinema* spp. AY274620 and AY274621 from coast of Mexico (Perez-Linares 2003).

The microscopic analysis of the *Oscillatoria* sp. UIO 017 showed great difference with other strains of Oscillatoriales and resembles *Phormidium* spp. In phylogenetic tree based on partial 16S rDNA-region red *Oscillatoria* sp. UIO 017 was found to be distantly related to the *Oscillatoria rosea* AB003164 (Ishida *et al* 1997). It is concluded that may be it belongs to another genus of Oscillatoriales.

It has mentioned previously that the identification of the strains belonging to the genera *Phormidium* and *Pseudoanabaena* spp. have various morphological characteristics in comparison with eachother and defining the *Phormidium* and *Pseudoanabaena* species have been problematic due to a variation of morphological features (Komárek and Anagnostidis 2005). The *Phormidium* spp. (UIO 018, UIO 145 and UIO 146) and *Pseudoanabaena* spp. (NIVA-CYA 280 and NIVA-CYA 333) strains studied here formed several stable clusters in the 16S rDNA gene tree. The strains of *Phormidium* UIO 018, UIO 145 and UIO 146 clustered with *Phormidium* sp. AB183567 from GenBank (Sekiguchi *et al* unpublished, Dobashi *et al* unpublished data). *Phormidium* spp. UIO 145, UIO 146 and UIO 018 were collected from Hulvika, Kaupang and Oslofjord, Norway, respectively and were 100% identical to eachother (as approved by phylogenetic trees of partial phycocyanin DNA and partial 16S rDNA-regions, in this study). This study shows the diversity of a single strain of *Phormidium* UIO 145, UIO 146 and UIO 018 in Norwegian waters (Lindstedt 1943, Wiik 1981, NIVA Culture Collection of Algae).

The combination of size of strains, habitat, molecular biological methods and microscopy made it possible to identify strains correctly (Neilan *et al* 1995, Rajaniemi-Wacklin *et al* 2008). No single method can provide correct identification of strains. Thus, it is likely that

species and strains that morphologically are quite similar, can be separated biochemically (Litvaitis 2002). The phylogenetic analysis is a reliable method to differentiate between toxic and non-toxic strains (Lyra *et al* 2001). Several workers have also suggested that the fatty acid composition of cyanobacteria appeared to be related to properties such as DNA (Holton *et al* 1968, Kenyon 1972). Kenyon (1972) worked on possible phylogentic significance of polyunsaturated fatty acids (PUFA) composition in the classification of Chroococcales.

Several strains of cyanobacteria have the ability to produce a range of bioactive compounds (Li *et al* 2001), of which the cyanotoxins are responsible for the death of variety of terrestrial and marine animals (Dow and Swoboda 2000). Finding of new strains from marine and brackish waters may be additional targets of bioactive compounds. Unfortunately, not much data are available from marine environments on cyanobacterial oligopeptides. In the present study three strains of *Synechococcus* UIO 015, *Pseudoanabaena* sp. UK-O-101 and *Geitlerinema* sp. UK-G-106 showed unique peaks of unknown oligopeptides (500-2000 Da) during LC-MS/MS analyses. The peaks of unknown oligopeptides indicate that the oligopeptides may be more common in marine waters than previously assumed (Carmichael and Li 2006). Domingos *et al* (1999) also reported that picoplankton *Synechococcus* spp. are known to produce microcystins. Carmichael and Li (2006) depicted that the *Synechococcus* spp. have potential to be a significant source of microcystin especially MC-LR and MC-YR in Salton Sea. The unknown oligopeptide of *Synechococcus* sp. UIO 015 and *Pseudoanabaena* UK-O-101 did not show any toxicity to *Artemia* nauplii. It means that both strains may contain on other oligopeptides, which don't have lethality to *Artemia*. There is a series of studies that attributed higher toxicity against *Daphnia* to other unknown compounds in permeate rather than to microcystins (Jungmann *et al* 1995, Jungmann and Benndorf 1994). *Geitlerinema* sp. UK-G-106 was only a single strain which showed toxicity to *Artemia,* both in crude and fractioned extracts.

Much work has been done on the genotypic diversity of *Geitlerinema* spp. (Margheri *et al* 2003, Sabrina *et al* 2007, McGregor and Rasmussen 2008, Krikwood *et al* 2008) but no work has been reported on its toxicity screening except only one report that presented the toxicity of *Geitlerinema unigranulatum* to Swiss mice (Mendonca 2006). During the study *Geitlerinema* sp. UK-G-106 showed significant toxicity to *Arteima* nauplii. The crude extract and MeOH:H_2O fractionations of *Geitlerinema* sp. UK-G-106 showed high toxicity to *Arteima* nauplii. The results represent that at the combined concentration (1, 0.1, 0.1, 0.02, 0.01, 0.01, 0.003. 0.002, 0.0016 and 0.001 mg dw mL^{-1}) of crude extract of *Geitlerinema* sp. UK-G-106 showed 0.0032 mg dw mL^{-1} $LC_{50,}$ which is lower than LC_{50} of *Planktothrix*

rubescens (0.05 mg dw mL^{-1}), *P. agardhii* (0.06 mg dw mL^{-1}) and *Microcystis aeruginosa* (0.12 mg dw mL^{-1}*).* Similarly, Feuillade *et al* (1996) and Jann-Para *et al* (2004) made studies with brine shrimp (*Artemia salina*) and found that the high toxic effects were observed in the crude extract of cyanobacteria *P. rubescens* that contained no detectable microcystins. In this study the crude extract of *Geitlerinema* sp. UK-G-106 showed high mortality of *Artemia* nauplii (0.0032 mg dw mL^{-1} LC_{50}) as compared to fractions of MeOH: H_2O (0.15-7.22 mg dw mL^{-1} LC_{50}). In the MeOH:H_2O fractions of *Geitlerinema* sp. UK-G-106, the highest mortality (0.15 mg dw mL^{-1} LC_{50}) was recorded in the water fraction, it also shows that the presence of a polar compound. Palikova *et al* (2007) worked on various fractions of cyanobacterial biomass (natural water blooms) with different composition and microcystin content on embryo larval development of carp (*Cyprinus carpio*) and found that the toxicity of fractioned extracts increases as concentration of MeOH decreases.

Geitlerinema sp. UK-G-106 was also tested for ability to induce cell death on rat promyelogenous leukaemia cells (IPC-81 wt). According to Linn Oftedal (personal communication) that 50% of the cell apoptosis (cell death) was observed but the death of cells were not caused by adenosine.

Much work has been done on detection of microcystins from freshwater strains of *Microcystis, Anabaena, Nostoc* and *Planktothrix* (Welker *et al* 2004a, b, Harada 2004, Harada *et al* 2004, Welker *et al* 2008, Welker and van Dohren 2006 and Dittmann and Wiegand 2006). A few studies focused on the isolation and detection of oligopeptides from marine genera i.e. *Synechococcus, Oscillatoria, Gleocapsa, Phormidium, Aphantheca* and *Lyngbya* (Carmichael and Li 2006). Nagle and Paul (1999) also worked on tropical marine mat forming strains of *Lyngbya majuscule, Hormothamnion, enteromorphoides* and several species of *Lyngbya, Schizothrix*, and *Microcoelus*. The presence of unknown oligopeptides in *Geitlerinema* sp. UK-G-106 is a new finding, which has not discovered before this study. More work is needed to extend this finding that the marine *Geitlerinema* sp. UK-G-106 has ability to produce oligopeptides.

It is also important to know that the collection site (near Astola Island, Pasni, Balochistan, Pakistan) of *Geitlerinema* sp. UK-G-106 is some 40 km away from East-south-East of the fishing port of Pasni. These waters are rich in variety of corals (personal communication with Dr. Rupert Ormond, Ex-Director, University of Marine Biological Station UMBS, Millport, Scotland during Cetacean Conservation Project at Astola Island) and receive heavy load of nutrients from river, port, city and villages. These eutrophic conditions may encourage algal blooms of *Geitlerinema* sp. UK-G-106. These blooms could possibly attack on corals that

contribute to the massive coral (directly), fish and avian (indirectly) mortalities. Recently, Richardson *et al* (2007) worked on black band disease (BBD) of coral reefs in relation with the presence of microcystins, anatoxin-a and saxitoxins in *Geitlerinema* sp. and *Leptolyngbya* sp. at Lee Stocking, Florida Keys, Island Bahamas, St. Croix and Philippines. They found that the both BBD *Geitlerinema* sp. and *Leptolyngbya* sp. cultures produced microcystin-LR. Myers *et al* (2007) suggested that the presence of microcystins in BBD plays a role in BBD pathology. The presence of *Geitlerinmea* sp. in the BBD microbial community and its ecological significance is harmful to coral reefs (Jorgensen *et al* 1979, Jorgensen 1982) and play an important role in terms of physiochemical structures of the bands (Richardson *et al* 2007). Perez-Linares (2003) have also reported the toxic bloom of *Geitlerinema* sp. and its toxicity associated to a mortality event of benthic fish and corals in the coast of Cabo San Lucas, Mexico. In the present study it is suggested that in Pakistani waters, the possible blooms and increase in mats formation of *Geitlerinema* sp. UK-G-106 can occur due to urban and rural pollution and they may harm coral reefs and their associated fauna and flora. The presence of possible toxins produced by the *Geitlerinema* sp. UK-G-106 may also produce higher amounts of toxin during favourable conditions in the natural habitat. There is a need for continuous monitoring of blooms of such species for further evaluation of the harmful effects of toxins on marine life.

This study is the first kind of its nature from marine waters, which indicates that Pakistani cyanobacterium *Geitlerinema* sp. UK-G-106 contains a new class of potent bioactive oligopeptide(s). This study shows that the *Geitlerinema* sp. UK-G-106 from Pakistani coastal waters is a promising source of new bioactive natural compounds that should be tested for further investigations, whether these can be used as a therapeutic drug in cancer treatment.

The present study will also act as a baseline for the future research of applied nature in Pakistan. It also provides information about biological diversity of cyanobacteria especially with regards to their ability to produce bioactive compounds and opens new area of research in Pakistan.

5. SUMMARY

• The growth media ES (30 PSU), IMR ½ (30 PSU) and Z8 (16 PSU) are best media for culturing of brackish and marine cyanobacterial strains.

• Microscopic analysis is not enough to identify strains.

• DNA sequence information of partial phycocyanin DNA-region (*cpc*BA) and partial 16S rDNA-region are useful tools to identify, investigate the presence of cyanobacteria, geographical distribution and their abundance in natural habitats.

• During partial 16S rDNA-region *Synechococcus* strains from Norway divided into 3 main clades.

• The phylogenetic analysis based on 16S rDNA verified the identification of studied strains. Cyanobacteria *Pseudoanabaena* spp. UIO 018, UIO 145 and UIO 146 were identified as *Phormidium* spp. UIO 018, UIO 145 and UIO 146, *Phormidium* sp. UIO 017 as *Oscillatoria* sp. UIO 017 and *Oscillatoria* sp. UK-G-106 as *Geitlerinema* sp. UK-G-106.

• The genetic profiles may provide a foundation for separating and quantifying genetically distinct groups of cyanobacteria in their natural habitats.

• There is a need to incorporate data from multiple sources (i.e. morphology, nucleotide sequences, biochemistry) in future revisions and new taxonomic descriptopns of cyanobacterial systematics.

• Among *Synechococcus* sp. UIO 015, *Pseudoanabaena* UK-O-101 and *Geitlerinema* sp. UK-G-106, only *Geitlerinema* sp. UK-G-106 showed remarkable toxicity to *Artemia* nauplii both in crude and fractioned extracts.

• The crude extract of *Geitlerinema* sp. UK-G-106 showed LC_{50}-24 h 0.0032 mg dw mL^{-1}.

• The fractionation with methanol:water of crude extract of *Geitlerinema* sp. UK-G-106 revealed that the mortality decreased as concentration of methanol increased.

• The LC_{50}-24 h of crude extract of *Geitlerinema* sp. UK-G-106 showed that the *Geitlerinema* sp. UK-G-106 was more toxic than toxic freshwater strains i.e. *Planktothrix rubescens* NIVA CYA 407, *P. agardhii* NIVA-CYA 229 and *Microcystis aeruginosa* NIVA-CYA 166.

• *Geitlerinema* sp. UK-G-106 was also sent to Linn Oftedal (University of Bergen) to test for ability to induce cell death on rat promyelogenous leukemia cells (IPC-81 wt). In this test 50% of the cell apoptosis (cell death) was observed. But according to Linn Oftedal the cell death observed was not caused by adenosine.

• The LC-MS/MS analysis showed the presence of unknown oligopeptides in 3 strains of marine cyanobacteria i.e. *Synechococcus* sp. UIO 015, *Pseudoanabaena* UK-O-101 and *Geitlerinema* sp. UK-G-106.

• Further experiments could not be continued as *Geitlerinema* sp. UK-G-106 is a dead strain.

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7. APPENDIX

Appendix 1. Recipes of growth media.

1. IMR 1/2 medium (70 %)

(Eppley *et al* 1967 modified by Paasche 1971)

Regents	**Stock solutions**	**mL per litre**
KNO_3	5 g/100 mL	0.5
KH_2PO_4	0.68 g/ 100 mL	0.5
Trace elements	Per litre	0.5
Na_2EDTA	6 g	
$FeCl_3.6H_2O$	1 g	
$MnSO_4.H_2O$	620 mg	
$ZnSO_4.7H_2O$	250 mg	
$Na_2MoO_4.2H_2O$	130 mg	
$CoCl_2.6H_2O$	4 mg	
$CuSO_4.5H_2O$	4 mg	
Vitamin solution		0.5
Thiamine (B-1)	10 mg/100 mL	
Cyanocobalamine (B-12)	0.1 mg/100 mL	
Biotine	0.1 mg/100 mL	
Filtered seawater (34 ‰)	700 mL	
Distilled water	300 mL	

•The seawater filtered by Whatman GF/C.
•The medium autoclaved for 15 minutes at 15 lb of pressure and 120 °C (Kawachi and Noël 2005).
•Adjusted the pH of the trace element stock solution to 8 with conc. NaOH
•Stock solution of vitamins were prepared in plastic bottles and stored at -20 °C.
•The medium kept at room temperature for one night.

2. Standard Z8 medium

(Staub 1961; modified Kotai 1972)

Regents	**Stock solutions (g/L)**	**mL per litre**
$NaNO_3$	46.7	10
$Ca(NO_3)_2.4H_2O$	5.9	10
$MgSO_4.7H_2O$	2.5	10
K_2HPO_4	3.1	10
$NaCO_3$	2.1	10
FeEDTA	15	10
Trace metal`s solution		1

FeEDTA: 10 mL $FeCl_3$ solution (2.8 g $FeCl_3.H_2O$ dissolved in 100 mL 0.1 N HCl) and 9.5 mL EDTA (3.9 g EDTA-Na_2 dissolved in 100 mL 0.1 N NaOH) are mixed and filled up to 1 litre distilled water.

Trace metals	**10 mL/L**
$Na_2WO_4.2H_2O$	0.33
$(NH_4)_6\ Mo_7O_{24}.4H_2O$	0.88
KBr	1.2
KJ	0.83
$ZnSO_4.7H_2O$	2.87
$Cd\ (NO_3)_2.4H_2O$	1.55
$Co\ (NO_3)2.6H_2O$	1.46
$CuSO_4.5H_2O$	1.25
$NiSO_4\ (NH_4)_2\ SO_4.6H_2O$	1.98
$Cr\ (NO_3)_3.9H_2O$	0.41
V_2O_5	0.089
$Al_2\ (SO_4)_3\ K_2SO_4.2H_2O$	4.74
	100 mL/L
H_3BO_3	3.1
$MnSO_4.4H_2O$	2.23

•When prepared media half of the distilled water was bubbled with air for 30 minutes to maintain pH up to 6.5-7.7.

3. Erd-Schreiber (ES) medium
(Provasoli 1968)

Regents	**Stock solutions**	**mL per litre**
$NaNO_3$	5 g/ 100 mL	2
$NaHPO_4.12H_2O$	1 g/ 100 mL	2
NaFeEDTA	150 mg/100 mL	0.5
Soil extract	100 mL/l	5
Vitamin solution	Per litre	2
Biotin	1 mg	
Thiamine	100 mg	
Cyanocobalamine (B-12)	1 mg	
Distilled water	1 litre	

•The seawater filtered by Whatman GF/C.
•Soil extract: one kg of fertile soil boiled in 1 litre distilled water. After cooling filtered the solution by an ordinary filter paper.
•Stored the soil extract in a fridge.
•Medium Pasteurized at 80 ºC for 15 minutes (Kawachi and Noël 2005).

4. Standard ASN III medium
(Rippka 1988)

Reagents	**Stock solsution (gm/L)**	**mL/litre**
NaCl	33	10
$MgCl_2.6H_2O$	2	10
KCl	0.5	10
$NaNO_3$	0.75	10
$K_2HPO_4.7H_2O$	0.02	10
$MgSO_4.7H_2O$	3.5	10
$CaCl_2.2H_2O$	0.5	10
Citric acid	0.003	10
Ferric ammonium citrate	0.003	10
EDTA (Disodium magnesium)	0.0005	5
Na_2CO_3	0.02	10

Trace elements		5
H_3BO_3	2.86	
$MnCl.4H_2O$	1.18	
$ZnSO_4.7H_2O$	0.222	
$Na_2MoO_4.2H_2O$	0.39	
$CuSO_4.5H_2O$	0.079	
$Co(NO_3)_2.6H_2O$	0.0494	
Distilled water	1 litre	

•The seawater filtered by an ordinary filter paper.
•Autoclaved for 20 minutes at 15 lb of pressure.
•Adjusted the pH to 7.5.

Appendix 2. Measurements of Norwegian cyanobacteria.

1. Chroococcus submarinus (Hansgirg) Kováčik NIVA-CYA 329

S. No.	Width of cell (µm)	Length of cell (µm)
1	16.1	16.3
2	18.2	20.2
3	18.7	19.7
4	19.3	20.0
5	18.7	19.2
6	19.1	19.6
7	18.4	20.0
8	19.3	19.2
9	23.9	23.3
10	24.7	25.4
11	24.1	24.2
12	25.1	25.7
13	24.0	24.3
14	24.5	24.6
15	24.8	25.8
16	23.1	23.1
17	20.3	21.9
18	19.1	20.2
19	21.7	23.3
20	18.2	20.1
21	23.9	24.3
22	24.0	24.1
23	20.0	21.0
24	17.0	17.1
25	20.0	20.9
26	23.0	23.0
27	16.8	16.9
28	19.5	19.6
29	20.8	20.8
30	23.5	24.1

2. Chroococcus submarinus **(Hansgirg) Kováčik NIVA-CYA 331**

S. No.	Width of cell (µm)	Length of cell (µm)
1	17.0	17.1
2	19.2	21.0
3	19.1	20.2
4	18.4	20.0
5	19.2	19.6
6	23.9	24.3
7	24.0	24.3
8	24.5	24.6
9	20.2	21.9
10	23.5	24.1
11	16.7	16.8
12	17.0	17.1
13	18.2	20.1
14	16.1	16.2
15	24.5	24.6
16	18.7	19.8
17	19.2	19.5
18	20.0	20.9
19	16.8	16.9
20	18.2	20.1
21	19.5	19.6
22	21.7	23.3
23	20.8	20.8
24	23.0	23.0
25	21.0	21.1
26	18.3	20.1
27	19.2	19.6
28	23.9	24.3
29	18.7	20.0
30	20.3	21.0

3. Synechococcus sp. Nageli NIVA-CYA 328

S. No.	Width of cell (µm)	Length of cell (µm)
1	0.9	1.9
2	0.9	1.8
3	0.6	0.6
4	0.5	1.4
5	0.5	0.9
6	0.7	1.4
7	0.9	1.5
8	1.0	1.5
9	0.8	1.4
10	0.8	1.8
11	0.7	1.3
12	0.6	0.6
13	1.0	1.3
14	0.9	1.5
15	0.8	1.1
16	1.0	1.8
17	0.5	1.1
18	0.5	0.5
19	0.7	1.5
20	1.3	0.6
21	1.0	1.7
22	0.9	1.6
23	0.7	1.3
24	0.9	1.6
25	0.5	1.0
26	0.9	1.4
27	0.5	1.8
28	0.8	0.8
29	0.6	2.0
30	0.6	1.4

***4. Spirulina subsalsa* Oersted ex Gomont NIVA-CYA 164**

S. No.	Width of trichome (µm)	Width of coil (µm)
1	3.3	3.9
2	3.0	4.1
3	3.2	3.2
4	3.3	4.0
5	3.9	3.8
6	3.4	4.3
7	3.4	3.9
8	3.2	3.5
9	3.4	3.7
10	3.6	4.1
11	3.5	3.5
12	3.5	3.5
13	4.1	3.5
14	3.7	3.3
15	3.7	3.4
16	3.4	3.6
17	4.0	3.5
18	4.0	3.7
19	3.6	3.8
20	3.5	4.0
21	3.5	3.7
22	3.4	3.7
23	3.3	3.8
24	3.6	3.5
25	3.2	3.9
26	3.4	3.8
27	3.8	3.8
28	3.4	4.0
29	3.5	3.8
30	3.2	3.7

***5. Pseudoanabaena* sp. Lauterborn NIVA-CYA 333**

S. No.	Width of cells (µm)	Length of cells (µm)
1	1.2	1.8
2	1.0	1.9
3	1.0	2.1
4	0.9	2.6
5	1.1	2.6
6	0.9	2.5
7	1.1	2.5
8	1.3	2.0
9	1.1	2.1
10	0.9	2.2
11	0.9	2.3
12	1.1	2.4
13	1.1	2.0
14	1.1	2.4
15	1.2	2.0
16	1.0	1.8
17	0.8	2.0
18	1.0	2.3
19	1.1	2.0
20	1.1	2.7
21	0.9	2.9
22	1.0	2.8
23	0.9	2.7
24	0.9	3.0
25	3.3	3.3
26	2.6	2.6
27	0.9	3.9
28	0.9	2.8
29	1.0	4.0
30	1.2	3.7

6. Spirulina subsala Oersted ex Gomont NIVA-CYA 163

S. No.	Width of trichome (µm)	Width of coil (µm)
1	3.5	3.4
2	3.3	3.4
3	3.3	3.6
4	3.4	3.4
5	3.6	3.5
6	3.5	3.3
7	3.9	3.3
8	3.5	3.4
9	3.4	3.5
10	3.5	3.2
11	3.3	3.8
12	3.6	3.7
13	3.5	3.8
14	4.1	3.8
15	3.6	3.4
16	3.6	3.7
17	3.6	3.2
18	3.7	3.6
19	3.6	3.4
20	3.3	3.5
21	3.4	3.4
22	3.6	3.9
23	3.5	3.4
24	3.2	3.6
25	3.4	3.6
26	3.3	3.8
27	3.1	3.5
28	3.1	3.5
29	3.6	3.4
30	3.6	3.4

***7. Oscillatoria* cf. *chalybea* (Merteens ex Gomont) Anagnostidis and Komárek NIVA-CYA 165**

S. No.	Width of cell (µm)	Length of cell (µm)
1	8.7	3.7
2	8.5	2.4
3	9.1	2.2
4	9.0	1.8
5	7.3	2.2
6	7.2	2.7
7	8.3	2.9
8	8.1	2.7
9	8.3	2.5
10	9.3	2.9
11	8.9	3.5
12	9.3	2.8
13	10.1	2.6
14	6.5	2.8
15	9.9	2.6
16	9.5	3.4
17	9.6	2.5
18	9.5	2.3
19	9.1	4.1
20	9.1	2.7
21	9.2	2.9
22	9.5	2.6
23	9.4	2.1
24	9.8	2.4
25	9.1	2.9
26	9.3	2.9
27	9.4	2.3
28	9.3	2.9
29	9.7	2.4
30	9.7	2.5

***8. Pseudoanabaena* sp. Lauterborn NIVA-CYA 280**

S. No.	Width of cell (µm)	Length of cell (µm)
1	0.5	2.5
2	0.3	2.1
3	0.5	2.1
4	0.6	1.9
5	0.6	2.0
6	0.7	2.1
7	0.6	1.9
8	0.6	1.6
9	0.6	2.2
10	0.4	2.6
11	0.5	2.5
12	0.1	3.0
13	1.0	2.3
14	0.9	2.1
15	0.6	2.2
16	0.4	2.2
17	0.9	2.5
18	0.4	2.3
19	0.4	1.8
20	0.4	2.0
21	0.4	1.9
22	0.2	2.1
23	0.3	2.3
24	1.1	2.5
25	0.9	2.2
26	0.5	1.6
27	0.7	2.0
28	0.7	2.7
29	0.6	2.5
30	0.5	2.0

***9. Phormidium* sp. Kützing ex Gomont UIO 018**

S. No.	Width of cell (µm)	Length of cell (µm)
1	1.2	1.7
2	0.9	2.7
3	1.2	2.1
4	1.2	1.6
5	1.2	1.7
6	0.6	1.8
7	1.0	1.3
8	0.9	2.3
9	0.9	2.6
10	1.1	2.2
11	1.1	2.4
12	1.1	3.3
13	1.5	2.4
14	1.2	2.8
15	1.2	2.6
16	1.1	2.5
17	1.1	2.7
18	1.4	2.9
19	1.1	1.9
20	1.1	1.7
21	1.2	2.4
22	1.0	3.0
23	1.0	3.1
24	1.2	3.1
25	1.0	2.9
26	0.7	2.8
27	1.1	1.8
28	1.1	2.3
29	1.4	2.3
30	1.2	2.4

***10. Oscillatoria* sp. Vaucher ex Gomont UIO 017**

S. No.	Width of cell (µm)	Length of cell (µm)
1	19.3	106.3
2	24.5	153.9
3	93.8	92.3
4	31.3	123.6
5	28.8	82.9
6	31.3	123.6
7	31.7	85.6
8	32.8	106.0
9	24.5	104.6
10	19.2	108.6
11	34.4	151.6
12	31.7	126.9
13	21.9	111.6
14	27.2	108.6
15	28.0	78.8
16	24.4	115.3
17	23.1	112.8
18	38.0	143.9
19	41.3	139.7
20	32.3	148.2
21	32.7	161.7
22	30.5	158.9
23	34.6	88.0
24	28.0	109.7
25	35.4	152.8
26	24.5	113.4
27	21.5	109.5
28	24.5	89.3
29	18.5	106.8
30	25.0	110.5

***11. Phormidium* sp. Kützing ex Gomont UIO 145**

S. No.	Width of cell (µm)	Length of cell (µm)
1	0.7	3.7
2	0.8	2.9
3	0.9	3.0
4	0.8	2.4
5	0.3	1.6
6	0.5	1.5
7	0.6	2.5
8	0.7	2.2
9	0.5	1.8
10	0.7	1.8
11	1.0	3.6
12	0.6	3.4
13	0.7	2.2
14	0.7	2.4
15	0.6	2.5
16	0.7	2.7
17	0.9	2.7
18	0.6	3.5
19	0.6	3.5
20	0.5	2.3
21	0.8	3.0
22	0.9	2.7
23	0.5	2.4
24	0.8	2.2
25	0.5	2.3
26	0.9	1.9
27	0.6	2.2
28	0.8	2.3
29	0.8	2.5
30	0.7	2.8

***12. Phormidium* sp. Kützing ex Gomont UIO 146**

S. No.	Width of cells (µm)	Length of cells (µm)
1	0.8	2.6
2	0.9	1.9
3	0.7	2.4
4	0.9	1.2
5	0.9	2.1
6	0.8	2.6
7	0.9	1.6
8	0.9	1.8
9	0.9	1.8
10	1.0	2.2
11	0.9	2.1
12	0.8	2.5
13	1.0	2.9
14	0.7	1.7
15	0.8	2.0
16	0.7	2.4
17	0.9	2.0
18	0.8	1.5
19	0.6	1.9
20	0.7	2.3
21	0.8	1.7
22	0.7	2.4
23	0.8	2.1
24	0.8	2.1
25	0.7	1.6
26	0.6	1.8
27	0.9	1.9
28	0.7	1.4
29	0.6	1.6
30	0.7	2.3

***13. Synechococcus* sp. Nageli UIO 015**

S. No.	Width of cells (µm)	Length of cells (µm)
1	0.9	0.9
2	0.5	0.7
3	0.8	0.9
4	0.7	0.7
5	0.4	0.7
6	0.7	0.8
7	0.6	0.6
8	0.6	0.8
9	0.5	0.8
10	0.5	0.7
11	0.7	0.7
12	0.4	0.7
13	0.5	0.5
14	0.3	0.3
15	0.5	0.5
16	0.5	0.7
17	0.5	0.5
18	0.7	0.7
19	0.3	0.3
20	0.3	0.8
21	0.3	0.7
22	0.4	0.4
23	0.5	0.7
24	0.4	0.7
25	0.7	0.8
26	0.5	0.6
27	0.5	0.8
28	0.4	0.6
29	0.4	0.6
30	0.5	0.5

***14. Synechococcus* sp. Nageli UIO 016**

S. No.	Width of cells (µm)	Length of cells (µm)
1	0.5	0.5
2	1.0	1.0
3	0.4	1.0
4	0.6	0.9
5	0.5	0.5
6	0.8	0.9
7	0.6	1.1
8	0.5	0.9
9	0.6	1.4
10	0.6	0.9
11	0.5	0.5
12	0.4	0.9
13	0.5	0.9
14	0.5	0.8
15	0.4	1.0
16	0.7	0.9
17	0.7	0.9
18	0.4	0.9
19	0.6	0.7
20	0.5	0.8
21	0.4	0.7
22	0.5	1.2
23	0.5	0.8
24	0.5	0.7
25	0.6	1.2
26	0.5	0.8
27	0.6	0.8
28	0.3	0.7
29	0.4	1.2
30	0.6	0.9

***15. Synechococcus* sp. Nageli UIO 013**

S. No.	Width of cells (µm)	Length of cells (µm)
1	0.4	0.6
2	0.4	0.5
3	0.3	0.4
4	0.3	0.9
5	0.4	0.6
6	0.4	0.4
7	0.4	0.4
8	0.4	0.6
9	0.4	0.4
10	0.9	0.9
11	0.4	0.4
12	0.4	0.7
13	0.7	0.9
14	0.6	0.9
15	0.5	2.1
16	0.3	0.6
17	0.5	0.5
18	0.7	0.6
19	0.7	0.9
20	0.6	0.6
21	0.7	0.9
22	0.5	1.0
23	0.5	0.6
24	0.3	0.7
25	0.6	0.6
26	0.7	1.0
27	0.6	0.6
28	0.5	0.5
29	0.5	0.6
30	0.4	0.5

***16. Synechococcus* sp. Nageli UIO 012**

S. No.	Width of cells (µm)	Length of cells (µm)
1	0.4	1.1
2	0.5	1.5
3	0.4	0.9
4	0.5	1.0
5	0.7	1.0
6	0.7	0.9
7	0.6	0.7
8	0.8	0.8
9	0.4	0.7
10	0.4	0.8
11	0.4	0.9
12	0.3	1.1
13	0.4	0.9
14	0.7	0.9
15	0.3	1.1
16	0.3	0.6
17	0.4	1.0
18	0.2	0.7
19	0.5	0.7
20	0.6	0.7
21	0.8	0.9
22	0.8	0.8
23	0.4	1.1
24	0.4	0.6
25	0.4	0.5
26	0.3	0.7
27	0.1	0.7
28	0.5	0.6
29	0.5	0.4
30	0.4	1.0

Appendix 3. Measurements of Pakistani cyanobacteria.

***17. Synechocystis* sp. Sauvageau UK-G-102**

S. No.	Width of cells (µm)	Length of cells (µm)
1	2.6	2.2
2	3.0	3.1
3	3.1	3.3
4	2.6	3.1
5	2.4	2.3
6	3.1	3.0
7	2.5	2.5
8	3.1	3.0
9	2.7	2.5
10	2.9	2.5
11	3.0	3.0
12	3.0	3.0
13	3.0	2.9
14	3.0	2.9
15	3.0	3.0
16	3.0	3.0
17	3.0	3.0
18	2.8	2.9
19	3.0	2.9
20	2.8	2.7
21	3.0	3.0
22	2.7	2.5
23	3.0	2.8
24	2.8	3.0
25	2.6	2.9
26	2.7	2.7
27	3.0	2.8
28	2.6	2.8
29	3.0	2.7
30	3.0	3.0

***18. Geitlerinema* sp. UK-G-106**
Anagnostidis (Anagnostidis et Komárek)

S. No.	Width of cells (µm)	Length of cells (µm)
1	7.9	4.6
2	10.5	4.1
3	9.5	4.9
4	8.1	4.3
5	8.5	4.6
6	7.2	5.1
7	8.7	5.1
8	10.1	3.2
9	10.3	4.2
10	10.9	4.1
11	9.8	4.4
12	7.8	4.2
13	8.7	4.4
14	9.7	4.9
15	9.7	3.5
16	10.4	3.7
17	8.2	4.3
18	7.9	3.6
19	9.5	2.7
20	10.1	3.2
21	8.8	4.1
22	6.3	3.7
23	4.5	4.3
24	5.4	5.2
25	8.3	4.3
26	6.7	3.9
27	9.2	4.6
28	8.9	4.7
29	8.8	3.4
30	6.5	5.2

***19. Chlorogleopsis* sp. Desikachary UK-O-105**

S. No.	Width of cells (µm)	Length of cells (µm)
1	2.0	2.3
2	1.3	2.9
3	1.5	1.7
4	1.4	1.8
5	1.4	3.4
6	2.0	2.3
7	1.5	1.8
8	1.5	1.9
9	2.0	2.2
10	1.5	1.6
11	1.4	3.3
12	1.4	3.2
13	2.0	2.4
14	1.7	1,76
15	1.5	2.3
16	2.0	1.7
17	1.8	1.5
18	1.6	1.7
19	1.8	1.5
20	1.4	1.8
21	1.7	2.7
22	2.0	2.1
23	1.9	2.0
24	2.0	1.9
25	2.0	2.1
26	2.1	2.0
27	2.9	2.1
28	1.4	1.8
29	2.1	2.0
30	2.1	1.8

***20. Pseudoanabaena* sp. Lauterborn UK-O-109**

S. No.	Width of cells (µm)	Length of cells (µm)
1	2.4	3.0
2	2.3	2.9
3	4.1	4.5
4	2.4	4.0
5	2.3	3.6
6	4.1	4.3
7	2.5	3.1
8	2.4	2.9
9	4.0	4.9
10	2.3	3.5
11	2.3	3.6
12	2.3	2.5
13	2.4	3.1
14	2.5	3.1
15	4.1	4.4
16	3.0	4.0
17	2.3	4.0
18	2.3	3.6
19	2.3	3.5
20	3.0	3.0
21	2.2	3.0
22	4.1	4.8
23	2.5	4.0
24	2.3	3.6
25	2.3	3.0
26	2.3	3.0
27	2.4	3.0
28	4.1	4.5
29	4.0	4.0
30	3.0	3.6

***21. Pseudoanabaena* sp. Lauterborn UK-O-101**

S. No.	Width of cells (µm)	Length of cells (µm)
1	2.0	3.0
2	2.1	3.1
3	2.0	3.5
4	1.8	3.2
5	1.9	3.3
6	2.0	3.1
7	2.0	2..97
8	2.2	3.5
9	2.0	3.5
10	2.0	3.1
11	2.0	3.5
12	2.1	3.6
13	2.1	3.0
14	2.1	3.1
15	2.2	3.1
16	2.1	3.8
17	2.0	4.0
18	2.1	3.6
19	2.1	3.0
20	2.1	3.1
21	2.0	3.0
22	2.2	3.0
23	2.1	3.7
24	2.0	3.5
25	2.0	3.1
26	3.0	3.1
27	2.2	2.1
28	2.0	3.5
29	1.9	3.1
30	1.9	3.0

***22. Oscillatoria* sp. Vaucher ex Gomont UK-G-110**

S. No.	Width of cells (µm)	Length of cells (µm)
1	3.2	4.0
2	3.5	5.6
3	3.1	4.3
4	3.6	7.0
5	3.4	6.4
6	2.9	6.0
7	3.0	3.4
8	3.5	6.5
9	3.1	4.3
10	3.6	7.0
11	3.5	6.4
12	3.5	5.6
13	3.1	4.0
14	3.5	5.5
15	3.0	4.3
16	3.6	6.0
17	3.5	6.4
18	3.5	5.8
19	3.2	4.0
20	3.7	5.7
21	3.1	4.4
22	3.6	6.8
23	3.5	6.5
24	3.6	5.6
25	3.1	3.9
26	3.5	5.5
27	3.0	4.3
28	3.6	6.0
29	3.4	6.4
30	3.5	5.6

***23. Chroococcus* sp. Nageli UK-G-103**

S. No.	Width of cells (µm)	Length of cells (µm)
1	4.7	5.6
2	5.2	5.5
3	6.5	6.5
4	4.5	4.6
5	5.1	5.8
6	4.5	4.6
7	4.7	5.5
8	5.2	5.6
9	6.0	6.9
10	5.5	5.6
11	5.2	5.7
12	4.5	4.6
13	5.4	5.6
14	5.1	5.5
15	5.5	5.5
16	5.4	4.9
17	5.4	5.8
18	5.9	5.7
19	5.7	5.6
20	5.2	5.5
21	6.4	6.8
22	5.7	5.9
23	5.3	5.8
24	6.1	6.0
25	4.7	5.7
26	5.5	5.5
27	6.1	6.5
28	4.4	4.5
29	5.2	5.7
30	5.5	5.7

***24. Oscillatoria* sp. Vaucher ex Gomont UK-G-108**

S. No.	Width of cells (µm)	Length of cells (µm)
1	3.0	2.7
2	3.7	4.1
3	3.1	4.1
4	3.7	2.9
5	3.7	4.2
6	3.5	2.8
7	3.0	4.3
8	3.2	2.5
9	3.8	4.2
10	2.9	4.0
11	3.8	2.5
12	4.0	4.1
13	3.9	2.5
14	3.6	2.8
15	3.2	2.9
16	3.0	2.6
17	3.3	3.2
18	3.5	3.9
19	3.6	3.8
20	3.7	3.8
21	3.3	2.6
22	3.4	3.2
23	1.9	2.6
24	3.5	2.6
25	4.0	3.0
26	3.8	2.7
27	3.0	2.4
28	3.9	2.4
29	1.9	2.3
30	3.8	2.7

Appendix. 4. Different sets of primers used in PCR reactions for different cyanobacterial species. (bold letters show successful results and used for DNA sequencing).

Serial no.	Strains	Primers to target 16S rDNA		Primerannealing	Primers to target cpcBA (phcocyanin DNA-region)		Primerannealing
		Forward	Reverse	Temperature (ºC)	Forward	Reverse	Temperature (ºC)
	Chroococcales						
1	*Chroococcus submarinus*	CYA 106 F	CYA 781R (a)	55	PCβF	PCαR	50
	NIVA-CYA 331	CYA 106 F	CYA 781R (b)	55	-	-	-
		CYA 359 F	CYA 781R (a)	50	-	-	-
		CYA 359 F	CYA 781R (b)	55	-	-	-
		CYA 106 F	CYA781R (b)	60	-	-	-
2	*Chroococcus submarinus*	CYA 106 F	CYA 781R (a)	55	PCβF	PCαR	50
	NIVA-CYA 329	CYA 106 F	CYA 781R (b)	55, 60	-	-	-
		CYA 359 F	CYA 781R (a)	50	-	-	-
		CYA 359 F	CYA 781R (b)	55	-	-	-
3	***Synechococcus* sp.**	**CYA 106 F**	**CYA 781R (a)**	**55**	PCβF	PCαR	45, 50
	NIVA-CYA 328	CYA 106 F	CYA 781R (b)	55	cpcBF	cpcAR	45
		CYA 359 F	CYA 781R (a)	50, 60	-	-	-
		CYA 359 F	CYA 781R (b)	55, 60	-	-	-
		CYA 106 F	CYA781R (b)	60	-	-	-
4	***Synechococcus* sp.**	**CYA 106 F**	**CYA781R (a)**	50, **55**, 60	PCβF	PCαR	45, 50
	UIO 012 sj. gr.	CYA 106 F	CYA781R (b)	50, 55, 60	cpcBF	cpcAR	45, 50
		CYA 359 F	CYA 781R (a)	50, 55, 60	-	-	-
		CYA 359 F	CYA 781R (b)	50, 55, 60	-	-	-
5	*Synechococcus* sp.	CYA 359 F	CYA 781R (b)	55	PCβF	PCαR	45, 50
	UIO 15 syn. UiB-15				cpcBF	cpcAR	45, 50
6	***Synechococcus* sp.**	**CYA 106 F**	**CYA 781R (a)**	**55**	PCβF	PCαR	45, 50
	UIO 016 syn. UiB-34	CYA 106 F	CYA 781R (b)	55, 60	cpcBF	cpcAR	45
		CYA 359 F	CYA 781R (a)	50, 60	-	-	-
		CYA 359 F	CYA 781R (b)	60	-	-	-
7	***Synechococcus* sp.**	**CYA 106 F**	**CYA 781R (a)**	**55**	**PCβF**	**PCαR**	45, **50**
	UIO 013 syn. Bl.gr	CYA 106 F	CYA 781R (b)	55	-	-	-
		CYA 359 F	CYA 781R (a)	50, 60	-	-	-
		CYA 359 F	CYA 781R (b)	55, 60	-	-	-
		CYA 106 F	CYA781R (b)	60	-	-	-
	Oscillatoriales						
8	*Oscillatoria cf. chalybea*	CYA106 F	CYA781R (a)	50, 52, 55, 58, 60	-	-	-
	NIVA-CYA 165	CYA106 F	CYA781R (b)	60	-	-	-
9	*Spirulina subsalsa*	CYA 106 F	CYA 781R (a)	55	PCβF	PCαR	45, 50
	NIVA-CYA 163	CYA 106 F	CYA 781R (b)	55, 60	-	-	-
		CYA 359 F	CYA 781R (a)	50, 60	-	-	-
		CYA 359 F	CYA 781R (b)	55, 60	-	-	-
					cpcBF	cpcAR	45, 50
10	*Spirulina subsalsa*	CYA 106 F	CYA 781R (a)	55	PCβF	PCαR	45, 50
	NIVA-CYA 164	CYA 106 F	CYA 781R (b)	55	-	-	-
		CYA 359 F	CYA 781R (a)	50, 60	-	-	-
		CYA 359 F	CYA 781R (b)	55, 60	-	-	-

Contd……

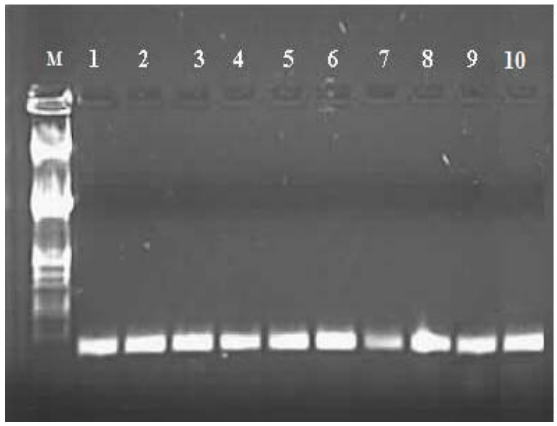
Contd. Table 4.

11	***Pseudoanabaena* sp.**	**CYA 106 F**	**CYA 781R (a)**	**55**	**PCβF**	**PCαR**	**50**
	NIVA-CYA 333	CYA 106 F	CYA 781R (b)	55, 60	-	-	-
		CYA 359 F	CYA 781R (a)	50, 60	-	-	-
		CYA 359 F	CYA 781R (b)	55, 60	-	-	-
12	***Pseudoanabaena* sp.**	**CYA 106 F**	**CYA 781R (a)**	**55**	-	-	-
	NIVA-CYA 280	CYA 106 F	CYA 781R (b)	55	-	-	-
		CYA 359 F	CYA 781R (a)	50, 60	-	-	-
		CYA 106 F	CYA781R (b)	60	-	-	-
		CYA 359 F	CYA 781R (b)	60	-	-	-
13	***Phormidium* sp.**	**CYA 106 F**	**CYA 781R (a)**	**55**	**PCβF**	**PCαR**	**50**
	UIO 018 syn. UiO-G	CYA 106 F	CYA 781R (b)	55, 60	-	-	-
		CYA 359 F	CYA 781R (a)	50	-	-	-
		CYA 359 F	CYA 781R (b)	55, 60	-	-	-
		CYA 106 F	CYA781R (b)	60	-	-	-
14	***Oscillatoria* sp.**	**CYA 106 F**	**CYA 781R (a)**	**55**	**PCβF**	**PCαR**	**50**
	UIO 017 syn. UiO-R	CYA 106 F	CYA 781R (b)	55, 60	-	-	-
		CYA 359 F	CYA 781R (a)	50, 60	-	-	-
		CYA 359 F	CYA 781R (b)	55, 60	-	-	-
					cpcBF	cpcAR	45, 50
15	***Phormidium* sp.**	**CYA 106 F**	**CYA 781R (a)**	**55**	**PCβF**	**PCαR**	45, **50**
	UIO 145 syn. Hulvika	CYA 106 F	CYA 781R (b)	55, 60	cpcBF	cpcAR	45
		CYA 359 F	CYA 781R (a)	50, 60	-	-	-
		CYA 359 F	CYA 781R (b)	55, 60	-	-	-
16	***Phormidium* sp.**	**CYA 106 F**	**CYA781R (a)**	50, **55**, 60	**PCβF**	**PCαR**	45, **50**
	UIO 146 syn. Kaupang	CYA 106 F	CYA781R (b)	50, 55, 60	cpcBF	cpcAR	45
		CYA 359 F	CYA 781R (a)	50, 55, 60	-	-	-
		CYA 359 F	CYA 781R (b)	50, 55, 60	-	-	-
17	***Geitlerinema* sp.**	**CYA106 F**	**CYA781R (a)**	50, 52, **55**, 58, 60	-	-	-
	UK-G-106	CYA106 F	CYA781R (b)	60	-	-	-

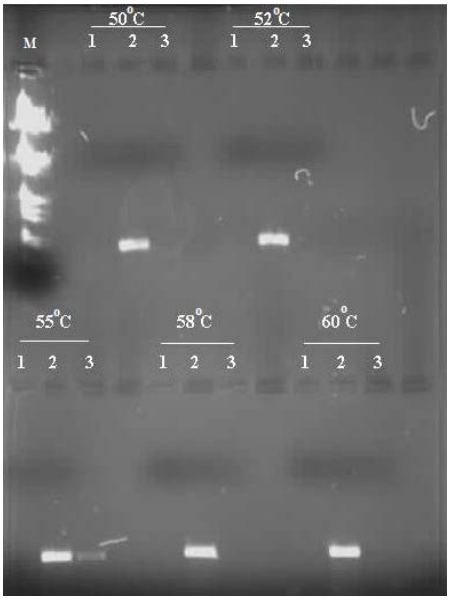
PCR temperature programme

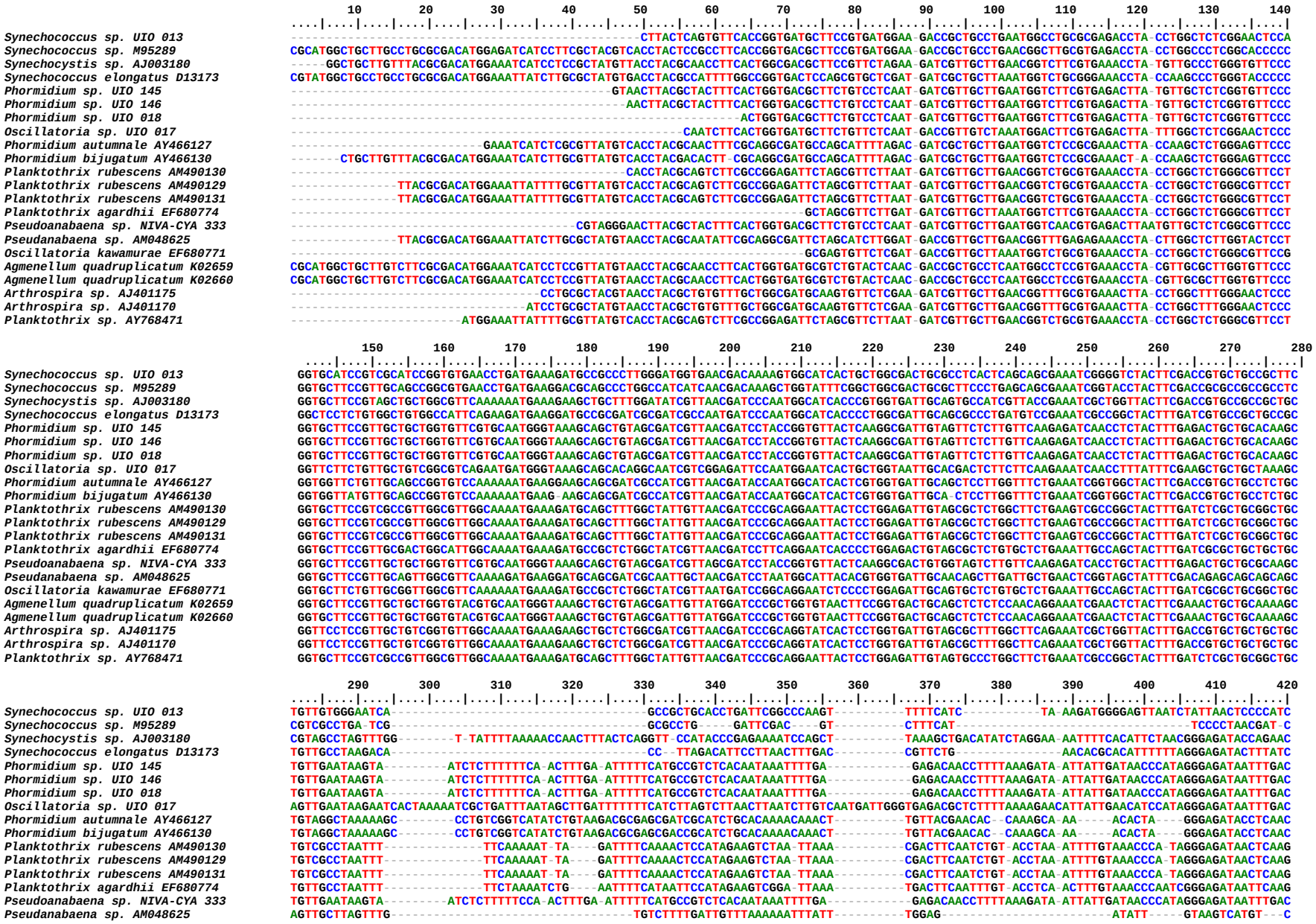
Denaturation of DNA at 94 ºC for 5 min
DNA synthesis and denaturation at 94 ºC for 1 min 35 cycles
Primerannealing for 1 min 35 cycles (for temp. see Table)
Extension at 72 ºC for 1 min 35 cycles
Extension at 72 ºC for 10 min

Appendix 5. PCR amplification bands of partial phycocyanin DNA-region (*cpc*BA) from Norwegian cyanobacterial strain at 55 °C primer annealing temperature. The bands in lane M is DNA size marker (Eco RI/Hind III) and 1-9 lanes correspond to the cyanobacterial strains *Synechococcus* sp. NIVA-CYA 328, *Synechococcus* sp. UIO 016, *Phormidium* sp. UIO 145, *Pseudoanabaena* sp. NIVA-CYA 333, *Pseudoanabaena* sp. NIVA-CYA 280, *Oscillatoria* sp. UIO 017, *Synechococcus* sp. UIO 013, *Synechococcus* sp. UIO 012, *Phormidium* sp. UIO 146 and lane 10 PCR water (negative control), respectively.

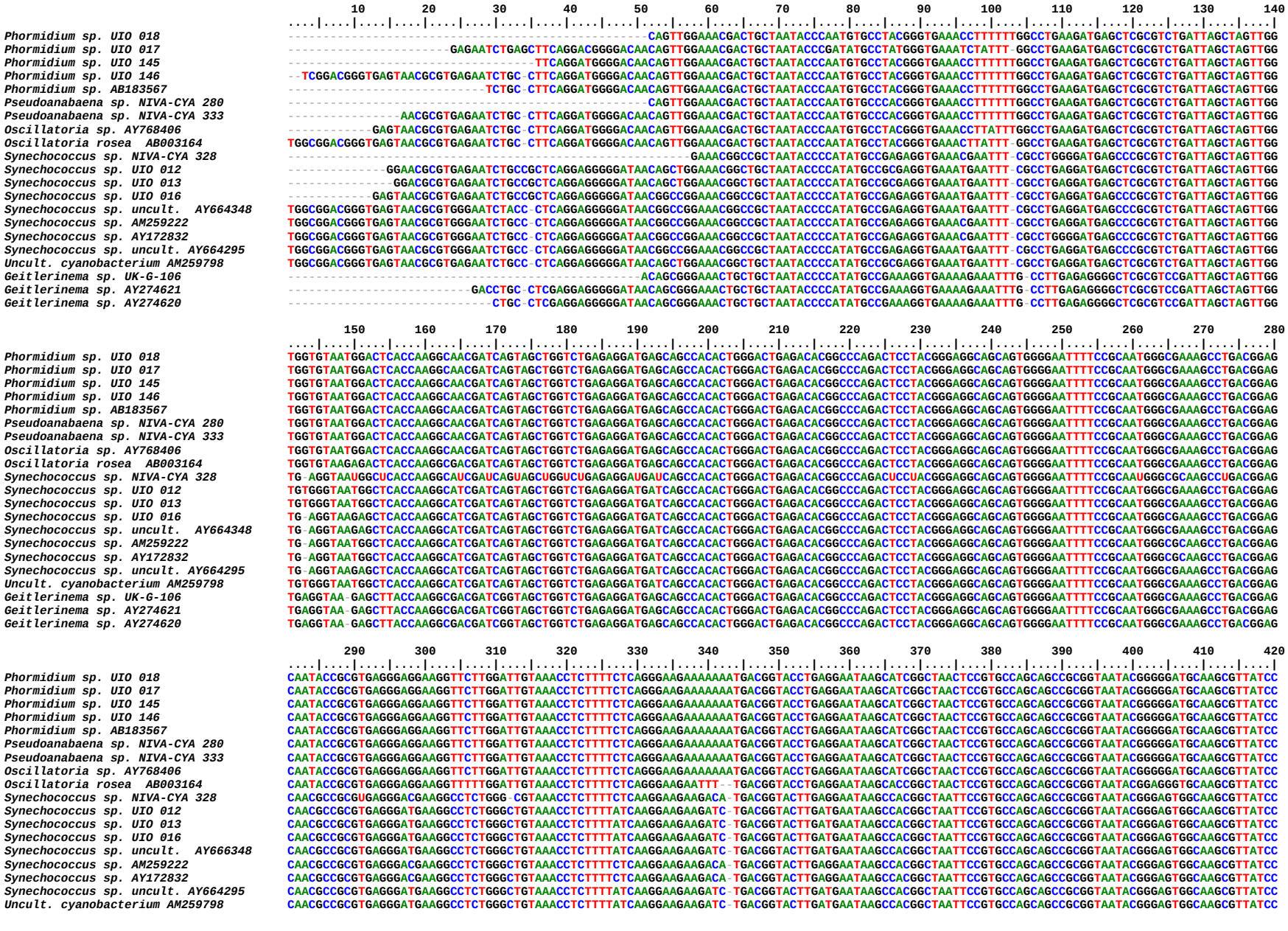


Appendix 6. PCR amplification bands of partial 16 rDNA-region Pakistani cyanobacterial strain at different primer annealing temperatures. The bands in lane M is DNA size marker (Eco RI/Hind III) and 1-2 lanes correspond to the cyanobacterial strains *Pseudoanabaena* sp. UK-O-101, *Geitlerinema* sp. UK-G-106 and lane 3 PCR water (negative control), respectively.

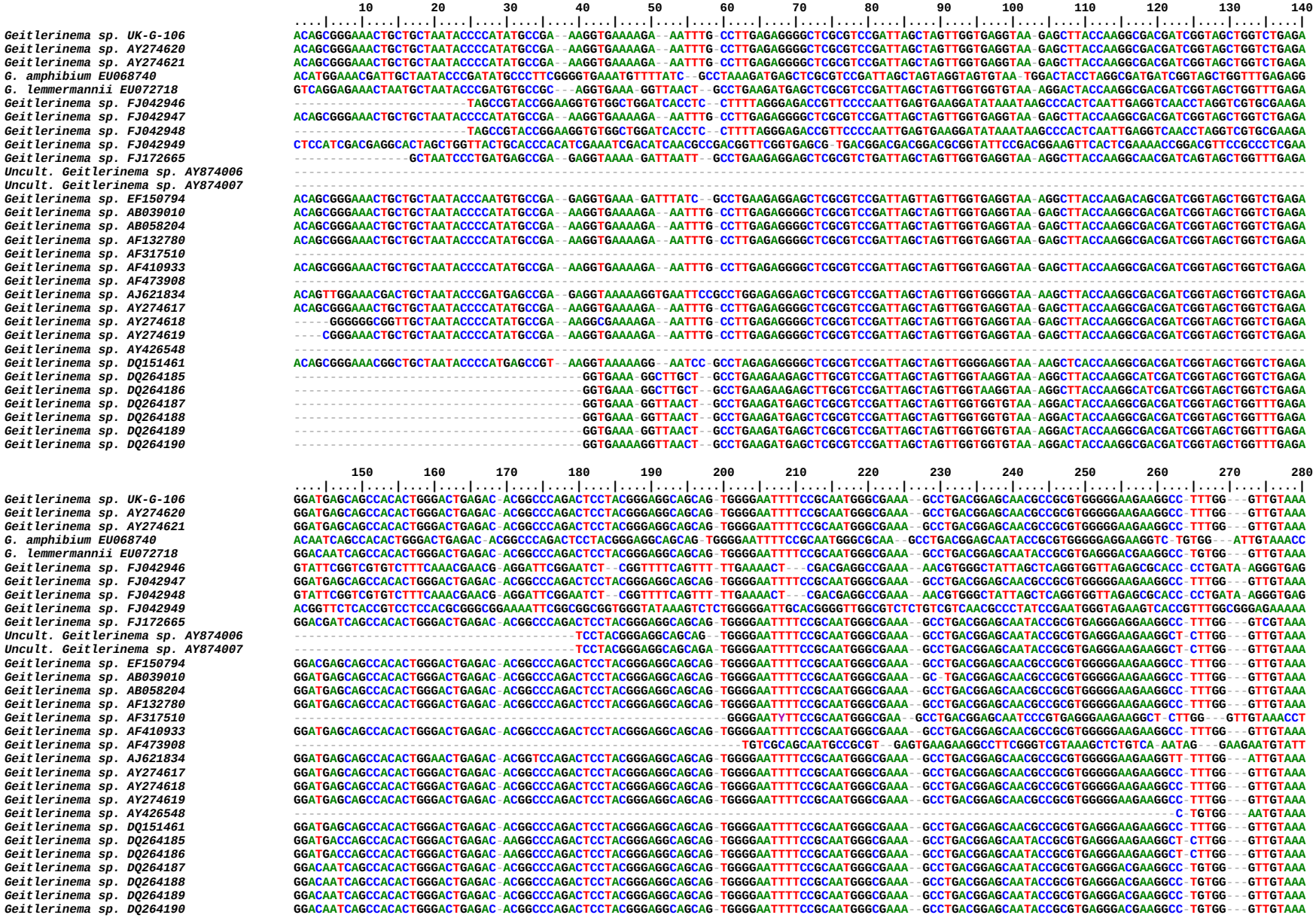


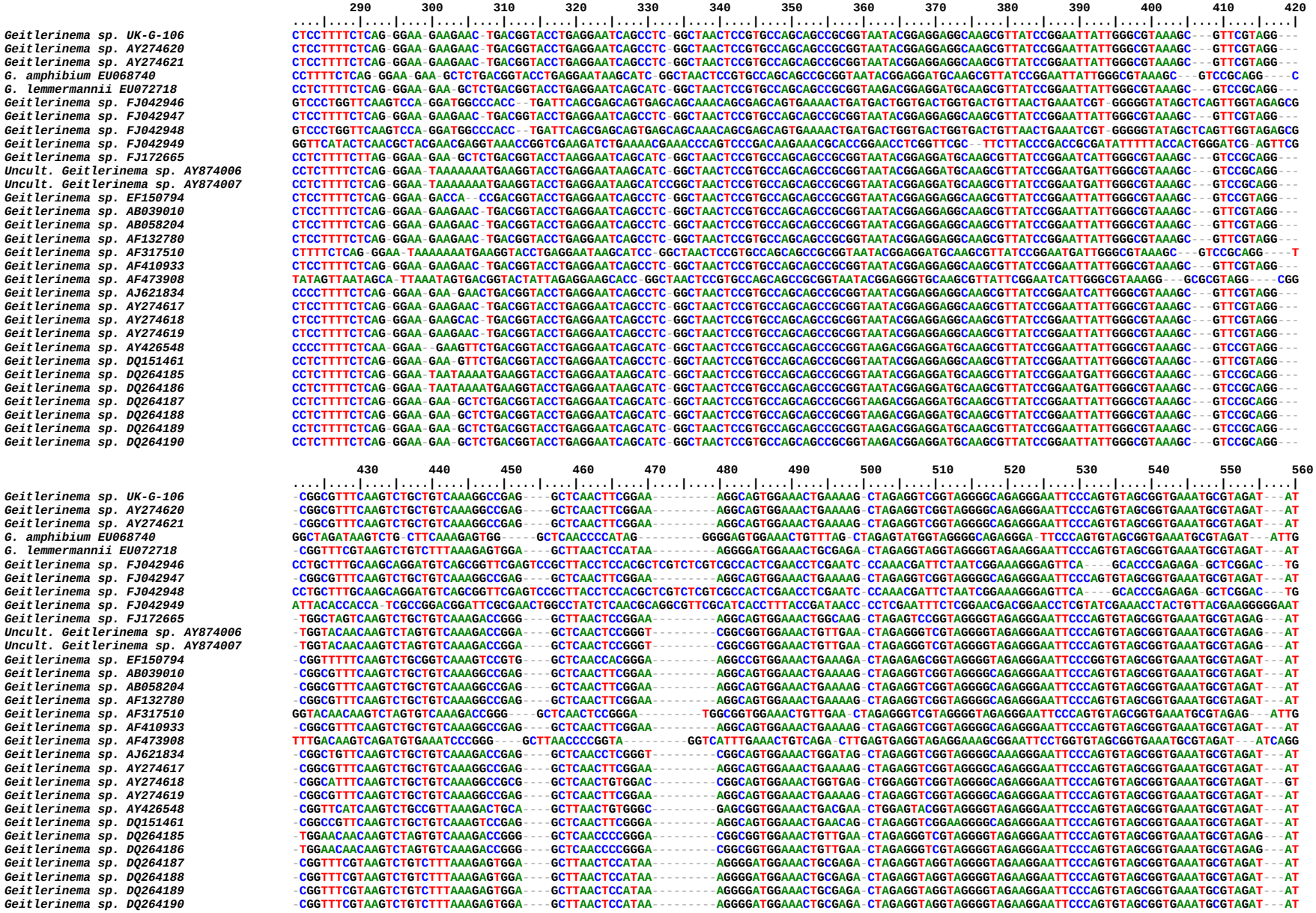

Synechococcus sp. UIO 013
Synechococcus sp. M95289
Synechocystis sp. AJ003180
Synechococcus elongatus D13173
Phormidium sp. UIO 145
Phormidium sp. UIO 146
Phormidium sp. UIO 018
Oscillatoria sp. UIO 017
Phormidium autumnale AY466127
Phormidium bijugatum AY466130
Planktothrix rubescens AM490130
Planktothrix rubescens AM490129
Planktothrix rubescens AM490131
Planktothrix agardhii EF680774
Pseudoanabaena sp. NIVA-CYA 333
Pseudanabaena sp. AM048625
Oscillatoria kawamurae EF680771
Agmenellum quadruplicatum K02659
Agmenellum quadruplicatum K02660
Arthrospira sp. AJ401175
Arthrospira sp. AJ401170
Planktothrix sp. AY768471

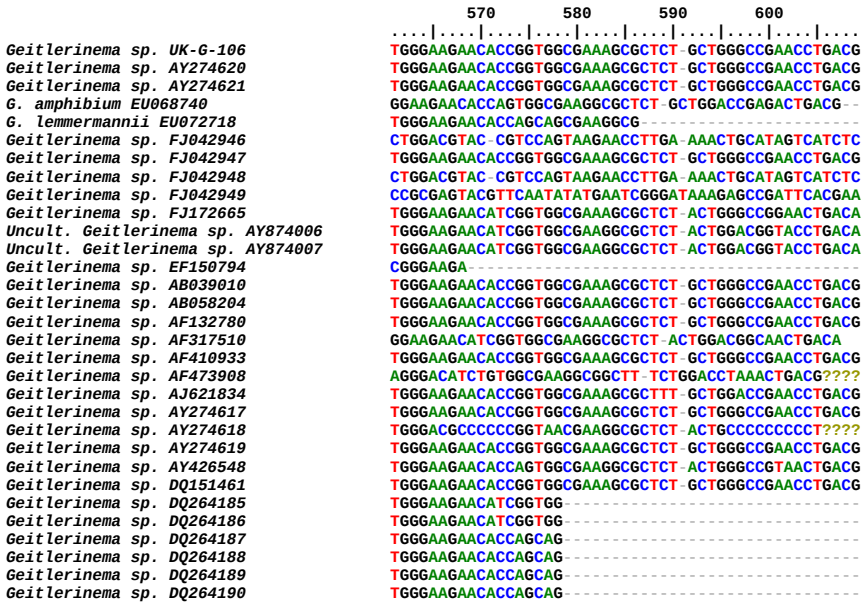
Appendix. 7. Alignment of the DNA sequences (partial phycocyanin DNA-region; *cpc*BA) of studied and their similar cyanobacterial strains from GenBank.


Phormidium sp. UIO 018
Phormidium sp. UIO 017
Phormidium sp. UIO 145
Phormidium sp. UIO 146
Phormidium sp. AB183567
Pseudoanabaena sp. NIVA-CYA 280
Pseudoanabaena sp. NIVA-CYA 333
Oscillatoria sp. AY768406
Oscillatoria rosea AB003164
Synechococcus sp. NIVA-CYA 328
Synechococcus sp. UIO 012
Synechococcus sp. UIO 013
Synechococcus sp. UIO 016
Synechococcus sp. uncult. AY664348
Synechococcus sp. AM259222
Synechococcus sp. AY172832
Synechococcus sp. uncult. AY664295
Uncult. cyanobacterium AM259798
Geitlerinema sp. UK-G-106
Geitlerinema sp. AY274621
Geitlerinema sp. AY274620

Appendix. 8. Alignment of the DNA sequences (partial 16S rDNA-region) of studied and their similar cyanobacterial strains from GenBank.


Geitlerinema sp. UK-G-106
Geitlerinema sp. AY274620
Geitlerinema sp. AY274621
G. amphibium EU068740
G. lemmermannii EU072718
Geitlerinema sp. FJ042946
Geitlerinema sp. FJ042947
Geitlerinema sp. FJ042948
Geitlerinema sp. FJ042949
Geitlerinema sp. FJ172665
Uncult. Geitlerinema sp. AY874006
Uncult. Geitlerinema sp. AY874007
Geitlerinema sp. EF150794
Geitlerinema sp. AB039010
Geitlerinema sp. AB058204
Geitlerinema sp. AF132780
Geitlerinema sp. AF317510
Geitlerinema sp. AF410933
Geitlerinema sp. AF473908
Geitlerinema sp. AJ621834
Geitlerinema sp. AY274617
Geitlerinema sp. AY274618
Geitlerinema sp. AY274619
Geitlerinema sp. AY426548
Geitlerinema sp. DQ151461
Geitlerinema sp. DQ264185
Geitlerinema sp. DQ264186
Geitlerinema sp. DQ264187
Geitlerinema sp. DQ264188
Geitlerinema sp. DQ264189
Geitlerinema sp. DQ264190


290 300 310 320 330 340 350 360 370 380 390 400 410 420
Geitlerinema sp. UK-G-106
Geitlerinema sp. AY274620
Geitlerinema sp. AY274621
G. amphibium EU068740
G. lemmermannii EU072718
Geitlerinema sp. FJ042946
Geitlerinema sp. FJ042947
Geitlerinema sp. FJ042948
Geitlerinema sp. FJ042949
Geitlerinema sp. FJ172665
Uncult. Geitlerinema sp. AY874006
Uncult. Geitlerinema sp. AY874007
Geitlerinema sp. EF150794
Geitlerinema sp. AB039010
Geitlerinema sp. AB058204
Geitlerinema sp. AF132780
Geitlerinema sp. AF317510
Geitlerinema sp. AF410933
Geitlerinema sp. AF473908
Geitlerinema sp. AJ621834
Geitlerinema sp. AY274617
Geitlerinema sp. AY274618
Geitlerinema sp. AY274619
Geitlerinema sp. AY426548
Geitlerinema sp. DQ151461
Geitlerinema sp. DQ264185
Geitlerinema sp. DQ264186
Geitlerinema sp. DQ264187
Geitlerinema sp. DQ264188
Geitlerinema sp. DQ264189
Geitlerinema sp. DQ264190
430 440 450 460 470 480 490 500 510 520 530 540 550 560
Geitlerinema sp. UK-G-106
Geitlerinema sp. AY274620
Geitlerinema sp. AY274621
G. amphibium EU068740
G. lemmermannii EU072718
Geitlerinema sp. FJ042946
Geitlerinema sp. FJ042947
Geitlerinema sp. FJ042948
Geitlerinema sp. FJ042949
Geitlerinema sp. FJ172665
Uncult. Geitlerinema sp. AY874006
Uncult. Geitlerinema sp. AY874007
Geitlerinema sp. EF150794
Geitlerinema sp. AB039010
Geitlerinema sp. AB058204
Geitlerinema sp. AF132780
Geitlerinema sp. AF317510
Geitlerinema sp. AF410933
Geitlerinema sp. AF473908
Geitlerinema sp. AJ621834
Geitlerinema sp. AY274617
Geitlerinema sp. AY274618
Geitlerinema sp. AY274619
Geitlerinema sp. AY426548
Geitlerinema sp. DQ151461
Geitlerinema sp. DQ264185
Geitlerinema sp. DQ264186
Geitlerinema sp. DQ264187
Geitlerinema sp. DQ264188
Geitlerinema sp. DQ264189
Geitlerinema sp. DQ264190



Appendix. 9. Alignment of the DNA sequences (partial 16S rDNA-region) of studied *Geitlerinema* sp. UK-G-106 and its similar *Geitlerinema* spp. from GenBank.

Appendix 10. Results of *Artemia franciscana* nauplii assay of positive controls, *Planktothrix rubescens, P. agardhii* and *Microcystis aeruginosa,* at different concentrations.

Planktothrix rubescens							***Planktothrix agardhii***							***Microcystis aeruginosa***						
No. of dead nauplii	**% of dead nauplii**	**Dilutions**	**Conc. (mg dw mL^{-1})**	**Conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**	**No. of dead nauplii**	**% of dead nauplii**	**Dilutions**	**Conc. (mg dw mL^{-1})**	**Conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**	**No. of dead nauplii**	**% of dead nauplii**	**Dilutions**	**Conc. (mg dw mL^{-1})**	**Conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**
39	97.5	1	2	2000	7.60090246	6.88	39	97.5	1	2	2000	7.6009025	6.88	37	92.5	0.5	2	2000	7.60090246	6.64
33	82.5	10	0.2	200	5.29831737	5.88	37	92.5	10	0.2	200	5.2983174	6.41	31	77.5	0.05	0.2	200	5.29831737	5.74
11	27.5	100	0.02	20	2.99573227	4.39	2	5	100	0.02	20	2.9957323	3.36	23	57.5	0.005	0.02	20	2.99573227	5.18
1	2.5	1000	0.002	2	0.69314718	3.36	1	2.5	1000	0.002	2	0.6931472	3.36	0	0	0.0005	0.002	2	0.69314718	0.2
LC_{50}: 0.05 mg dw mL^{-1}			3.903497 x value	49.5755119 exp value			**LC_{50}: 0.06 mg dw mL^{-1}**			4.1426154 x value	62.96729093 exp value			**LC_{50}: 0.12 mg dw mL^{-1}**			4.7954598 x value	120.9599864 exp value		

Appendix 11. Results of *A. franciscana* assay of *Geitlerinema* sp. at different concentrations.

No. of dead nauplii	**% of dead nauplii**	**Dilutions**	**Conc. (mg dw mL^{-1})**	**Conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**	**No. of dead nauplii**	**% of dead nauplii**	**Dilutions**	**Conc. (mg dw mL^{-1})**	**Conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**	**No. of dead nauplii**	**% of dead nauplii**	**Dilutions**	**Conc. (mg dw mL^{-1})**	**Conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**
38	95	1	1	1000	6.907755279	6.64	33	82.5	10	0.1	100	4.605170186	5.92	38	95	1	1	1000	6.907755279	6.64
34	85	10	0.1	100	4.605170186	5.92	30	75	50	0.02	20	2.995732274	5.67	34	85	10	0.1	100	4.605170186	5.92
25	62.5	100	0.01	10	2.302585093	5.31	30	75	100	0.01	10	2.302585093	5.67	33	82.5	10	0.1	100	4.605170186	5.67
1	2,5	1000	0.001	1	0	3.36	29	72.5	300	0.003	3	1.098612289	5.58	30	75	50	0.02	20	2.995732274	5.67
							23	57.5	500	0.002	2	0.693147181	5.18	25	62.5	100	0.01	10	2.302585093	5.58
							21	52.5	600	0.0016	1.6	0.470003629	5.05	30	75	100	0.01	10	2.302585093	5.92
														29	72.5	300	0.003	3	1.098612289	5.18
														23	57.5	500	0.002	2	0.693147181	5.05
														21	52.5	600	0.0016	1.6	0.470003629	5.31
														1	2,5	1000	0.001	1	0	3.36
LC_{50}: 0.016 mg dw mL^{-1}			2.776553548 x value	16.06356314 exp value			**LC_{50}: 0.00047 mg dw mL-1**			-0.746341463 x value	0.474097888 exp value			**LC_{50}: 0.0032 mg dw mL^{-1}**			1.173567406 x value	3.233507321 exp value		

Appendix 12. Fractionations of extract (50:50; MeOH:H_2O) of *Geitlerinema* sp. for *A. franciscana* assay at 0%, 20%, 40%, 60%, 80% and 100% MeOH.

0% MeOH						
No. of dead nauplii	**% of dead nauplii**	**Dilution**	**conc. (mg dw mL^{-1})**	**conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**
33	82.5	1	1	1000	6.90775528	5.92
13	32.5	10	0.1	100	4.60517019	4.39
8	20	100	0.01	10	2.30258509	4.16
4	10	1000	0.001	1	0	3.72
1	2.5	10,000	0.0001	0.1000	-2.3025851	2.97
LC_{50}: 0.15 mg dw mL^{-1}					4.9947423 x value	147.634895 exp value

20% MeOH						
No. of dead nauplii	**% of dead nauplii**	**Dilution**	**conc. (mg dw mL^{-1})**	**conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**
31	77.5	1	1	1000	6.90775528	5.74
13	32.5	10	0.1	100	4.60517019	4.53
3	7.5	100	0.01	10	2.30258509	3.52
2	5	1000	0.001	1	0	3.36
0	0	10,000	0.0001	0.1000	-2.30258509	0
LC_{50}: 0.17 mg dw mL^{-1}					5.16 x value	174.164456 exp value

40% MeOH						
No. of dead nauplii	**% of dead nauplii**	**Dilution**	**conc. (mg dw mL^{-1})**	**conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**
27	67.5	1	1	1000	6.90775528	5.44
10	25	10	0.1	100	4.60517019	4.33
3	7.5	100	0.01	10	2.30258509	3.52
2	5	1000	0.001	1	0	3.36
0	0	10,000	0.0001	0.1000	-2.30258509	0
LC_{50}: 0.25 mg dw mL^{-1}					5.5479984 x value	256.723184 exp value

60% MeOH						
No. of dead nauplii	**% of dead nauplii**	**Dilution**	**conc. (mg dw mL^{-1})**	**conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**
16	40	1	1	1000	6.90775528	4.75
2	5	10	0.1	100	4.60517019	3.36
2	5	100	0.01	10	2.30258509	3.36
1	2.5	1000	0.001	1	0	2.95
0	0	10,000	0.0001	0.1000	-2.3025851	0
LC_{50}: 1.36 mg dw mL^{-1}					7.2188661 x value	1364.94048 exp value

80% MeOH						
No. of dead nauplii	**% of dead nauplii**	**Dilution**	**conc. (mg dw mL^{-1})**	**conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**
7	17.5	1	1	1000	6.90775528	4.05
6	15	10	0.1	100	4.60517019	3.96
2	5	100	0.01	10	2.30258509	3.36
1	2.5	1000	0.001	1	0	2.95
0	0	10,000	0.0001	0.1000	-2.30258509	0
LC_{50}: 2.21 mg dw mL^{-1}					7.7022244 x value	2213.265709 exp value

100% MeOH						
No. of dead nauplii	**% of dead nauplii**	**Dilution**	**conc. (mg dw mL^{-1})**	**conc. (ng dw mL^{-1})**	**ln (ng dw mL^{-1})**	**Probit values**
4	10	1	1	1000	6.90775528	3.72
2	5	10	0.1	100	4.60517019	3.52
2	5	100	0.01	10	2.30258509	3.36
1	2.5	1000	0.001	1	0	2.95
0	0	10,000	0.0001	0.1000	-2.30258509	0
LC_{50}: 7.22 mg dw mL^{-1}					8.8847369 x value	7220.914602 exp value

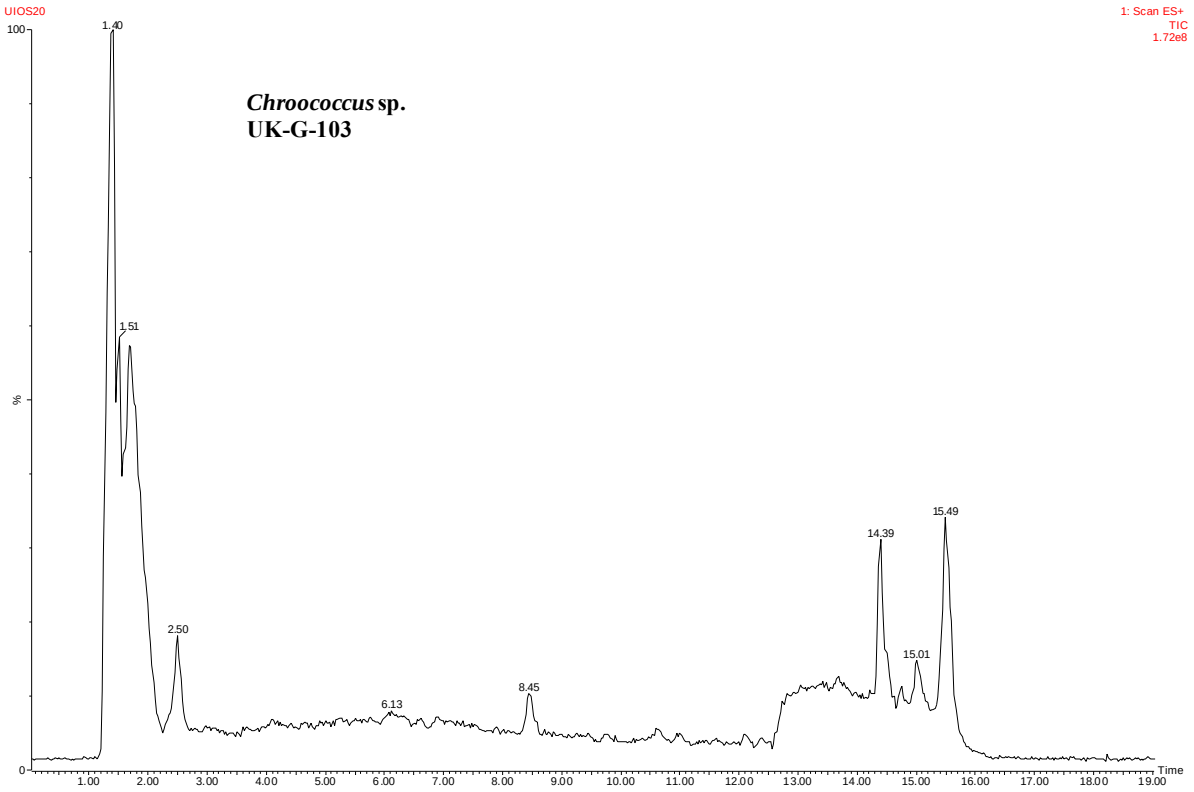
Appendix 13. Concentration and lethality of standard toxin, sodium dodecyl sulphate (SDS) for *Artemia* nauplii.

Serial no.	**SDS** (mg/L)	**Volume SDS** (0.01%) mL	**Volume** (70% seawater mL)
1	10	1	9
2	13.5	1.35	8.65
3	18	1.8	8.2
4	24	2.4	7.6
5	32	3.2	6.8

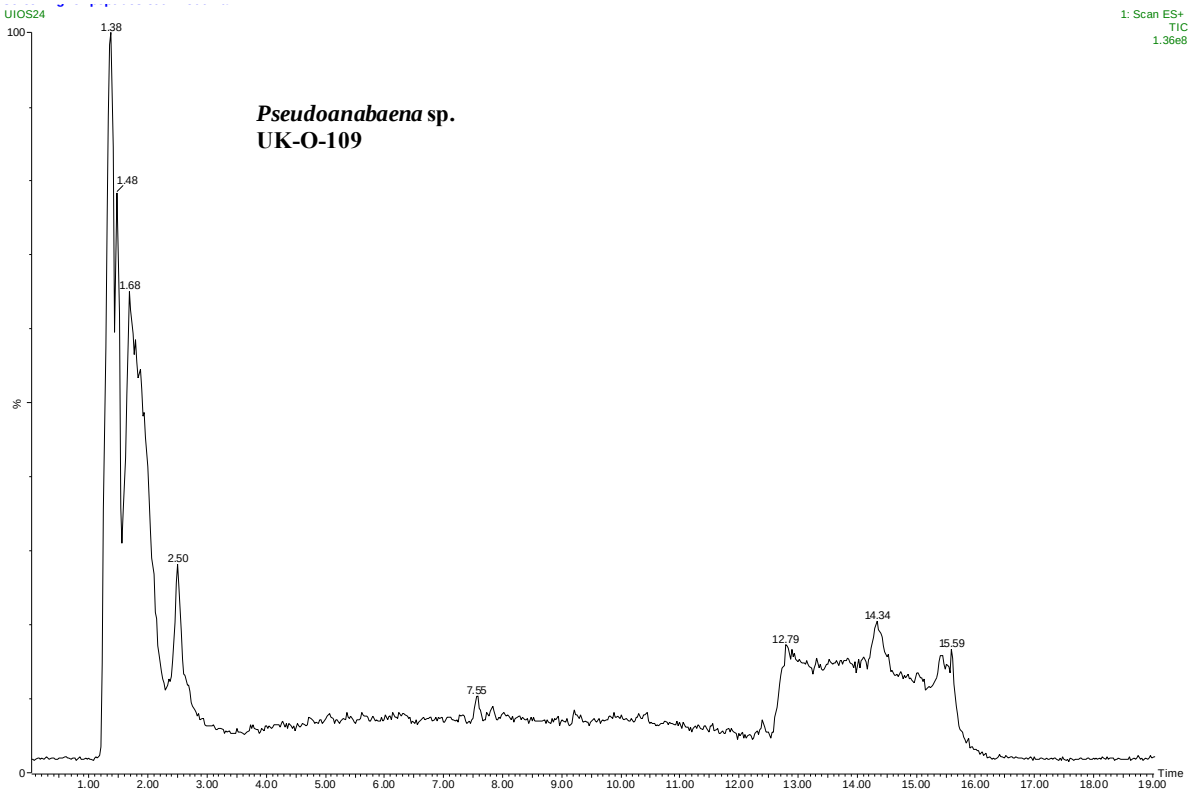
Appendix 14. Results of *A. franciscana* assay of SDS at different concentrations.

No. of dead nauplii	**% of dead nauplii**	**Conc. (ng mL^{-1})**	**ln (ng mL^{-1})**	**Probit values**
39	97.5	32000	10.37349118	6.88
33	82.5	24000	10.08580911	5.92
23	57.5	18000	9.798127037	5.18
8	20	13500	9.510444964	4.16
1	2.5	10000	9.210340372	2.95
LD_{50}: 17.85 mg mL^{-1}				
		9.7901844 x value	17857.59881 exp value	

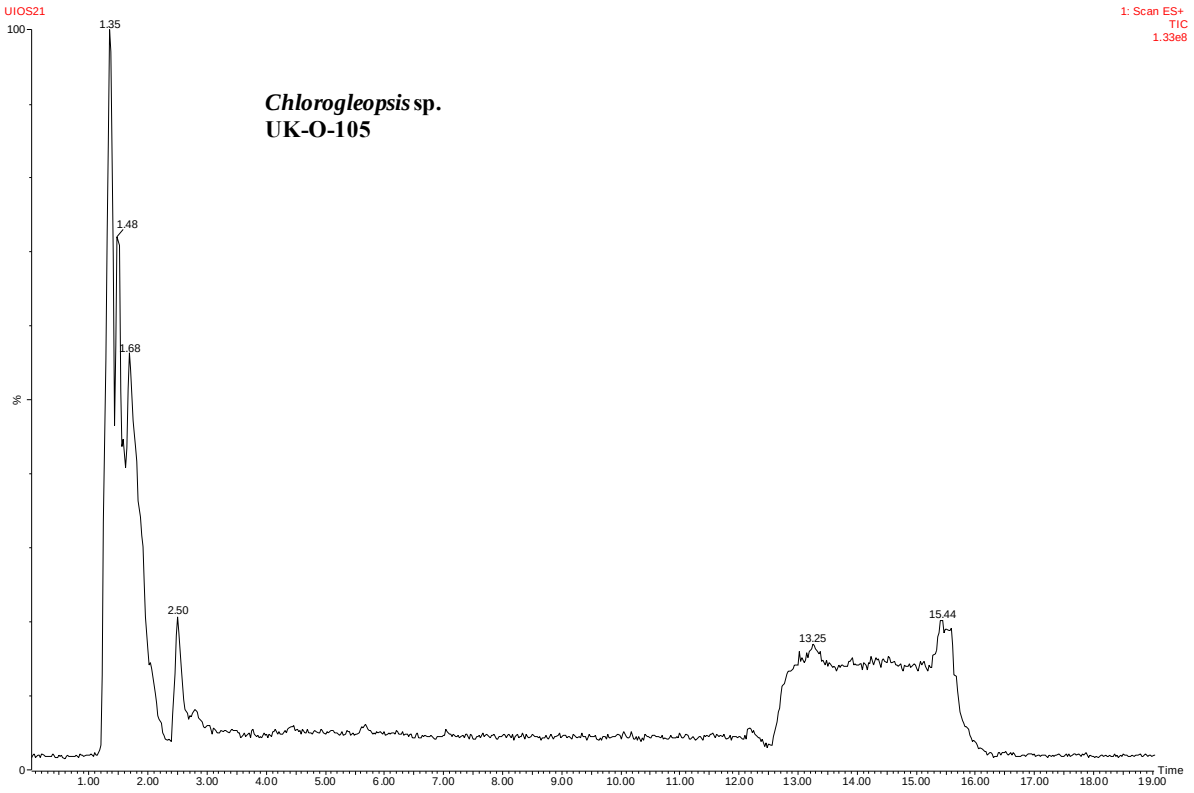
Appendix. 15. Chromatograms of cyanobacterial strains by LC-MS-MS.



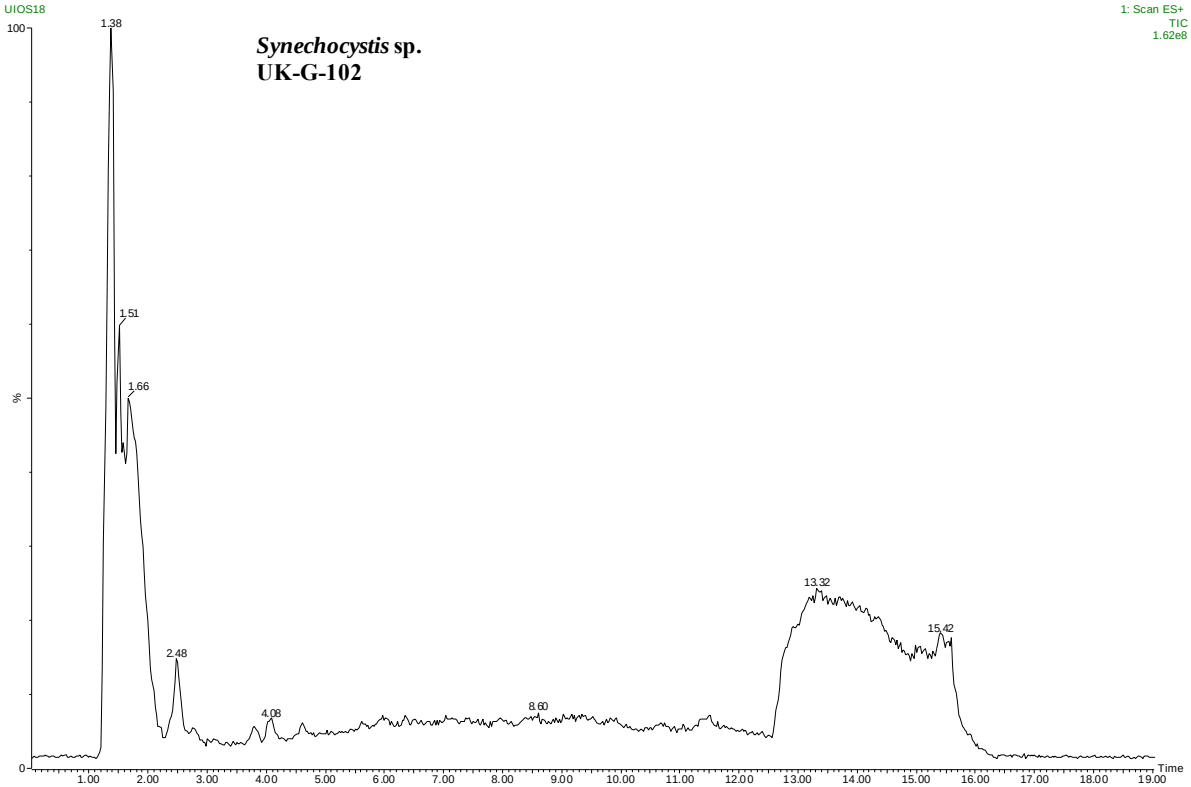
***Chroococcus* sp.**
UK-G-103



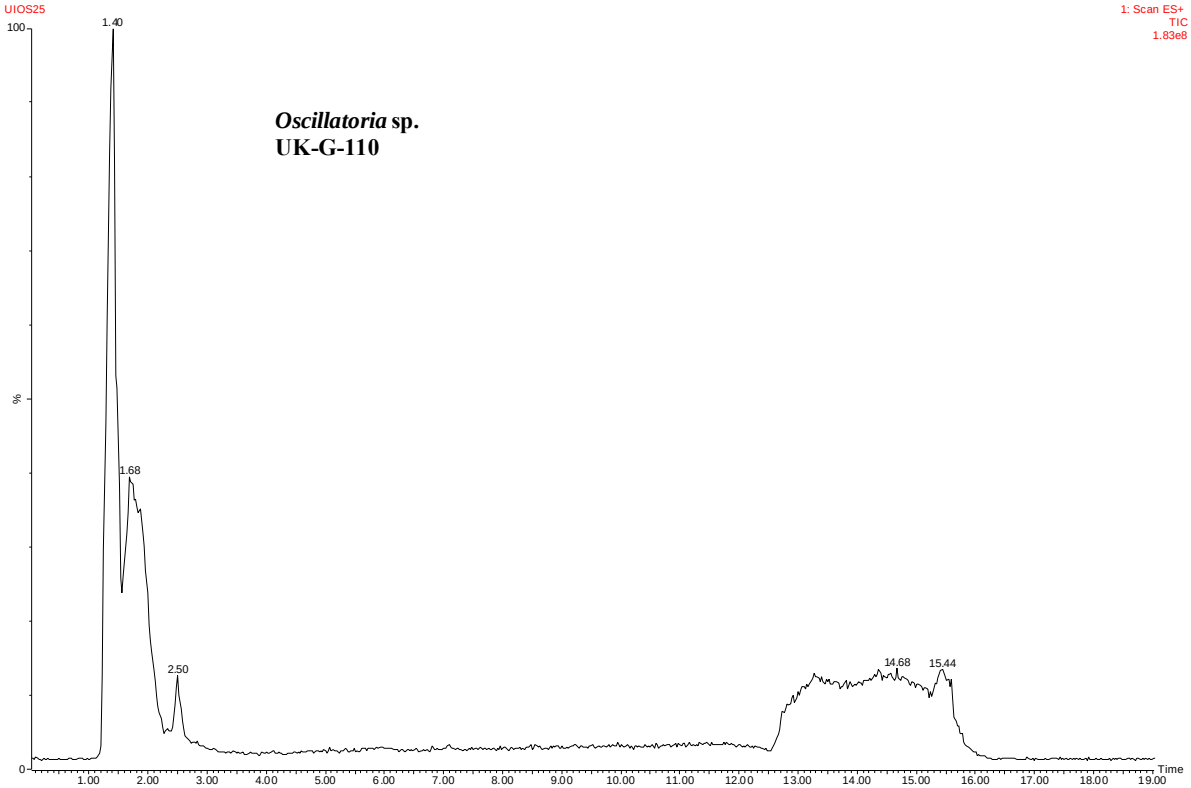
***Pseudoanabaena* sp.**
UK-O-109



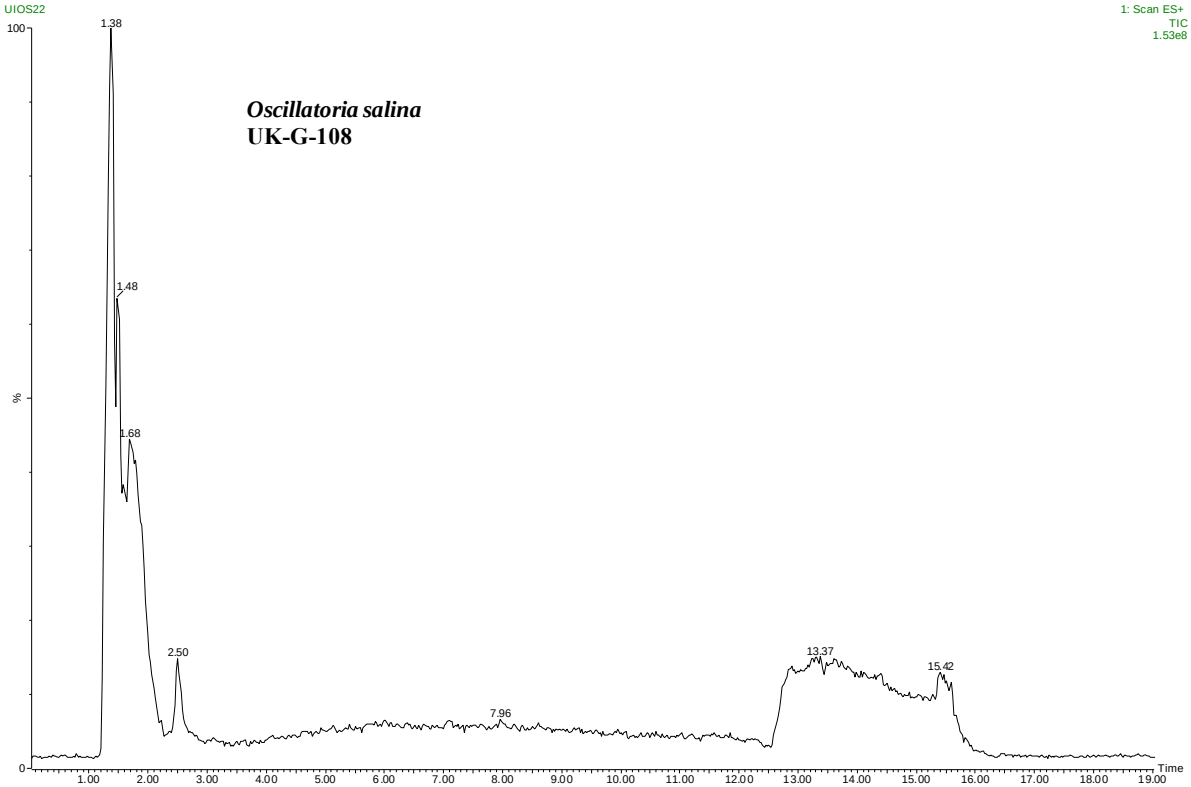
***Chlorogleopsis* sp.**
UK-O-105



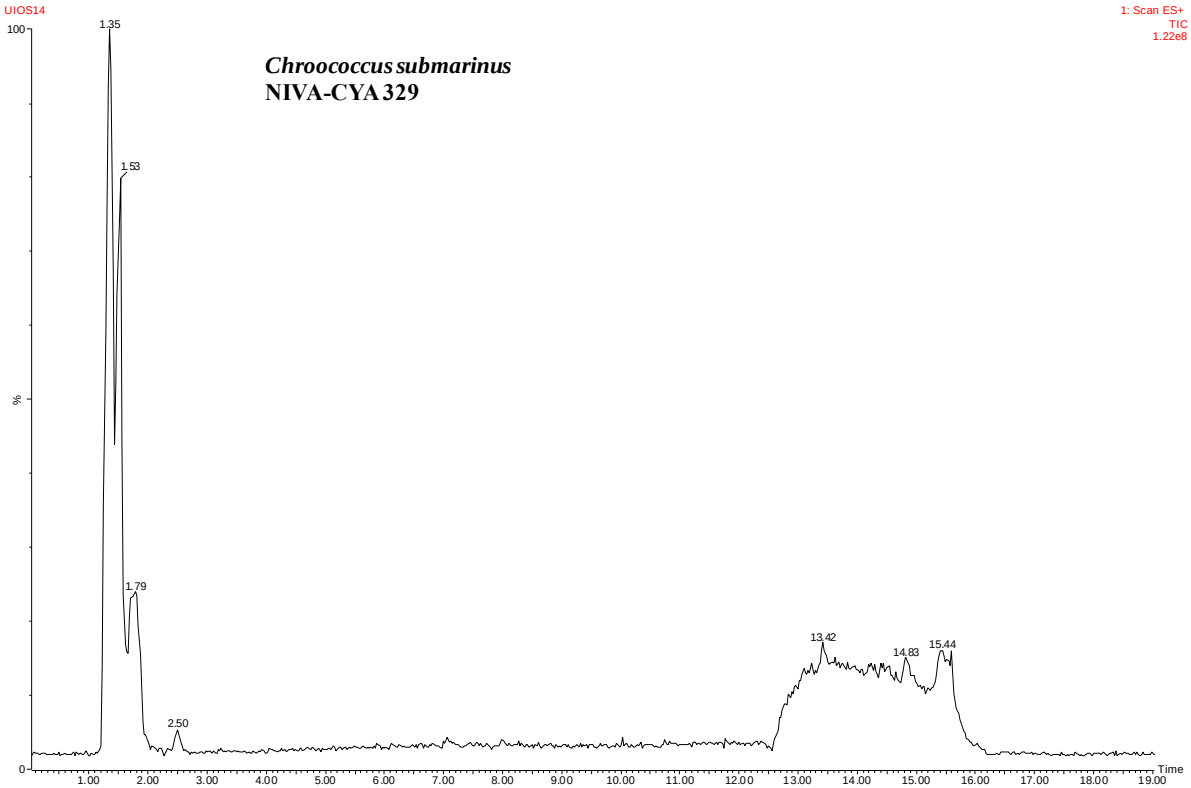
***Synechocystis* sp.**
UK-G-102



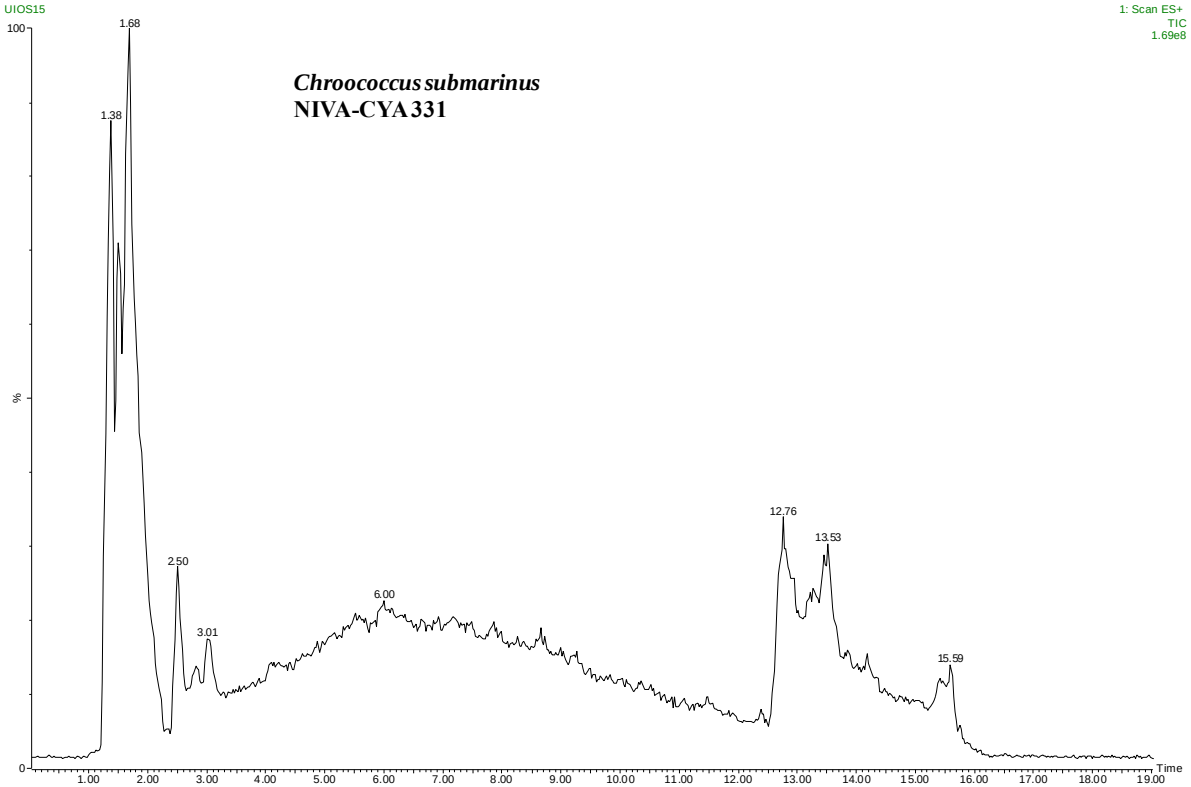
***Oscillatoria* sp.**
UK-G-110



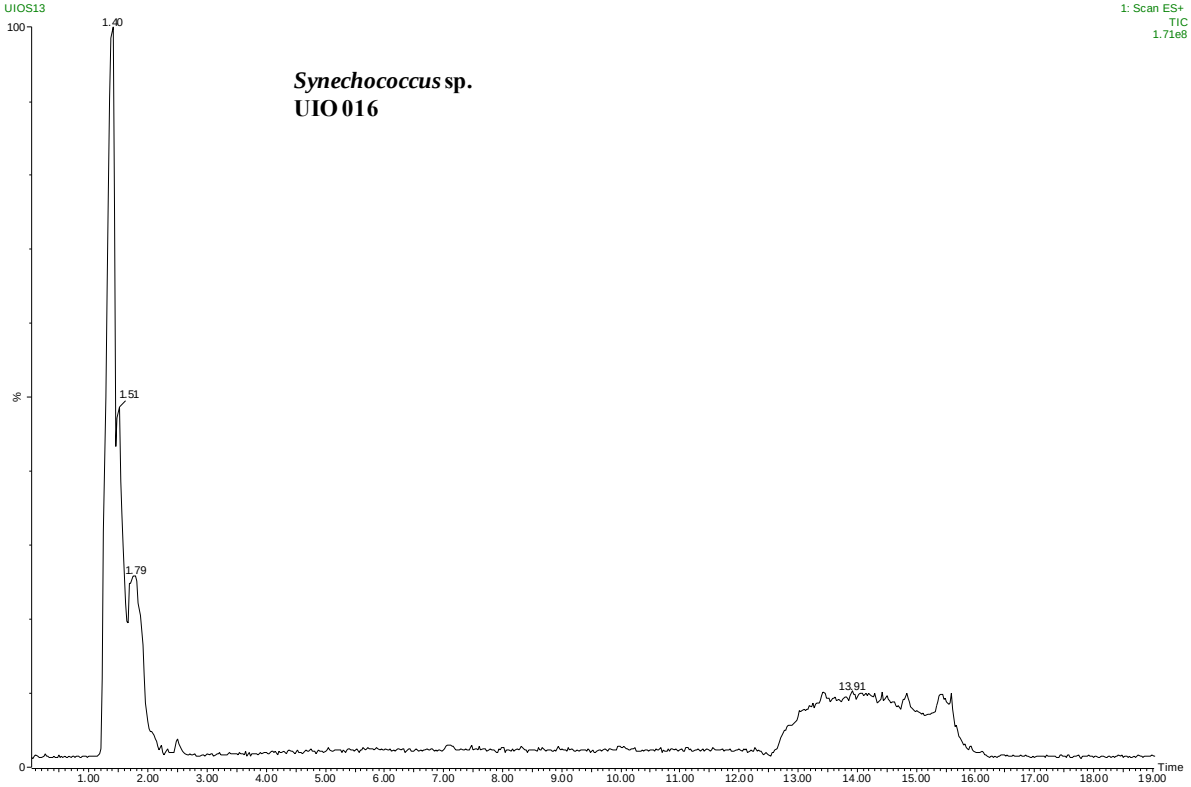
Oscillatoria salina
UK-G-108



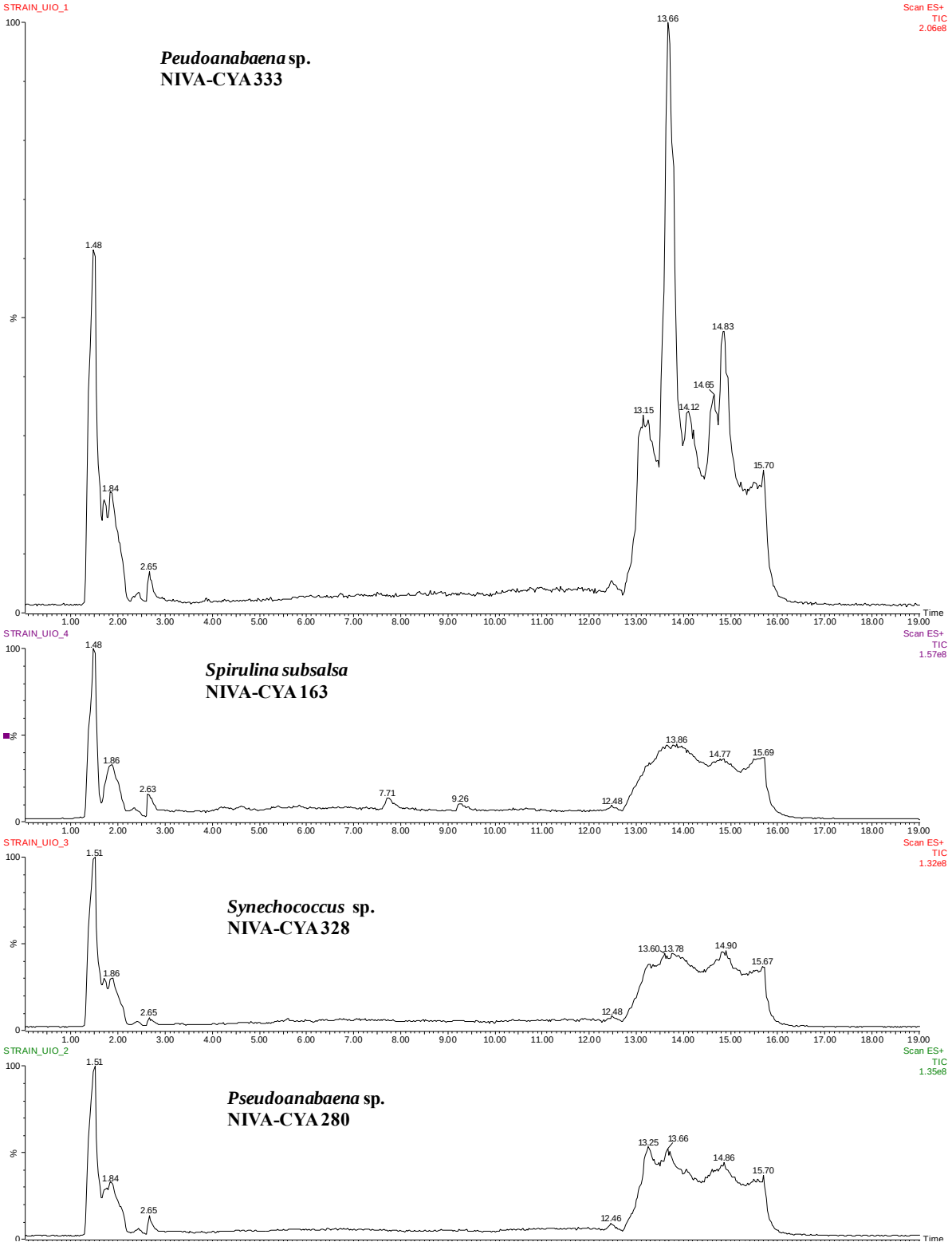
Chroococcus submarinus
NIVA-CYA 329

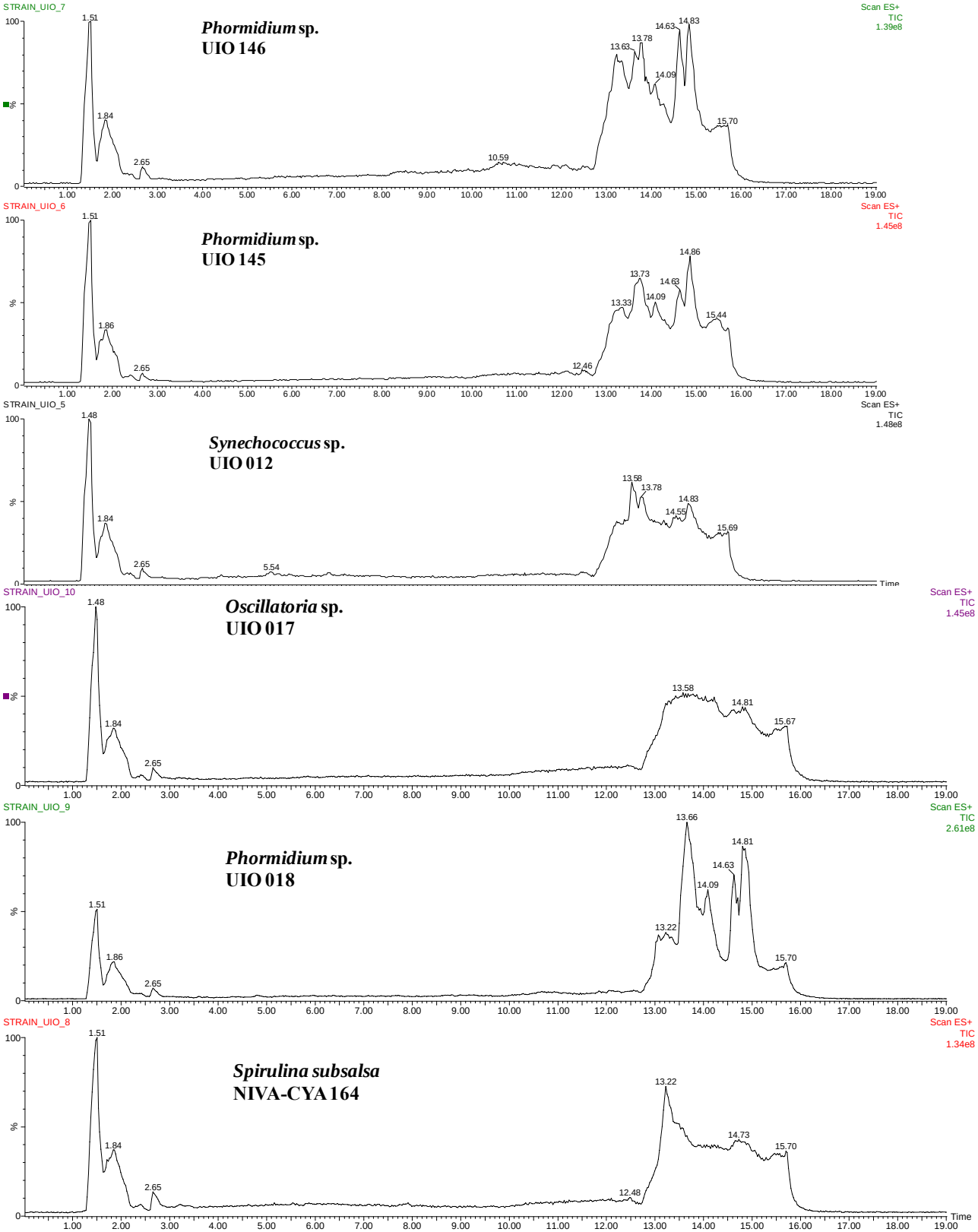


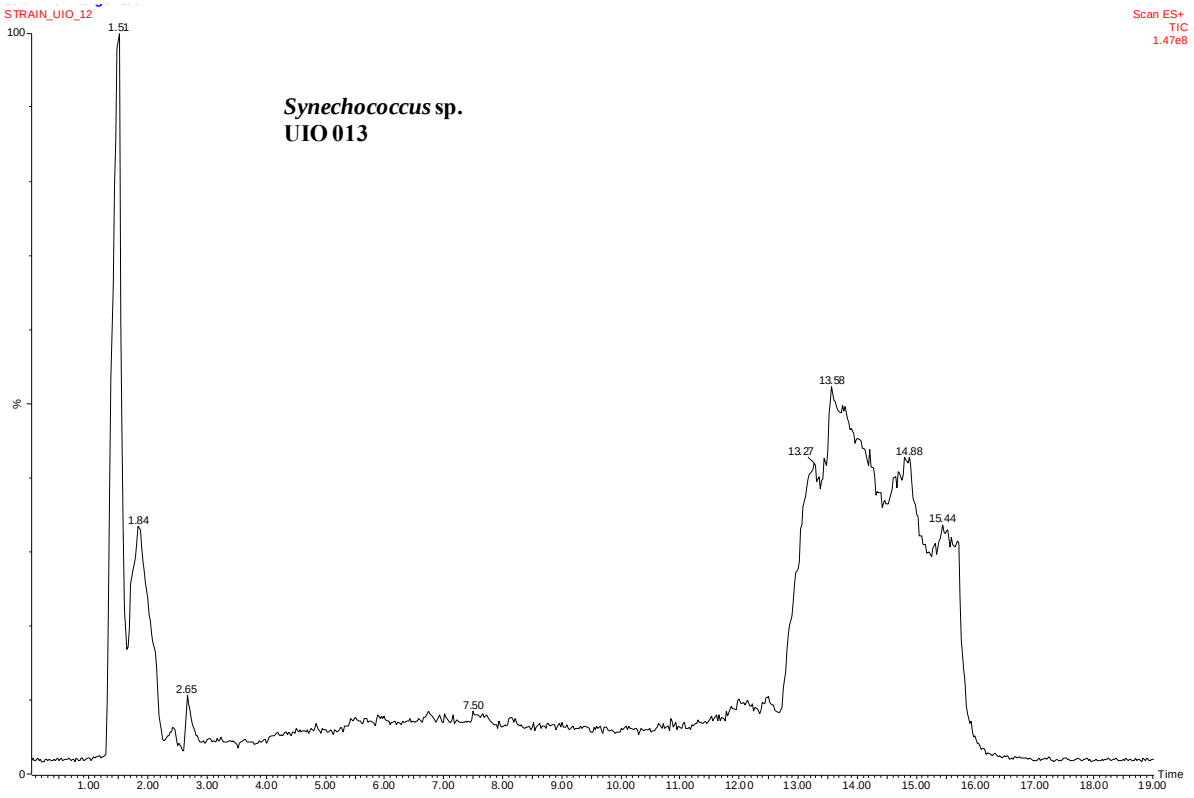
Chroococcus submarinus
NIVA-CYA 331



***Synechococcus* sp.**
UIO 016


STRAIN_UIO_1
Scan ES+
TIC
2.06e8
Peudoanabaena sp.
NIVA-CYA 333
1.48
1.84
2.65
13.15
13.66
14.12
14.65
14.83
15.70
Time
STRAIN_UIO_4
Scan ES+
TIC
1.57e8
Spirulina subsalsa
NIVA-CYA 163
1.48
1.86
2.63
7.71
9.26
12.48
13.86
14.77
15.69
STRAIN_UIO_3
Scan ES+
TIC
1.32e8
Synechococcus sp.
NIVA-CYA 328
1.51
1.86
2.65
12.48
13.60
13.78
14.90
15.67
STRAIN_UIO_2
Scan ES+
TIC
1.35e8
Pseudoanabaena sp.
NIVA-CYA 280
1.51
1.84
2.65
12.46
13.25
13.66
14.86
15.70
Time


STRAIN_UIO_7
Scan ES+
TIC
1.39e8
Phormidium sp.
UIO 146
STRAIN_UIO_6
Scan ES+
TIC
1.45e8
Phormidium sp.
UIO 145
STRAIN_UIO_5
Scan ES+
TIC
1.48e8
Synechococcus sp.
UIO 012
STRAIN_UIO_10
Scan ES+
TIC
1.45e8
Oscillatoria sp.
UIO 017
STRAIN_UIO_9
Scan ES+
TIC
2.61e8
Phormidium sp.
UIO 018
STRAIN_UIO_8
Scan ES+
TIC
1.34e8
Spirulina subsalsa
NIVA-CYA 164
Time



***Synechococcus* sp.**
UIO 013